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(54) Title: OLIGONUCLEOTIDE MEDIATED INHIBITION OF HEPATITIS B VIRUS AND HEPATITIS C VIRUS REPLICATION

(57) Abstract: The present invention relates to nucleic acid molecules, including antisense and enzymatic nucleic acid molecules, such as hammerhead ribozymes, DNAzymes, Inozymes, Zinzymes, Amberzymes, and G-cleaver ribozymes, which modulate the synthesis, expression and/or stability of an HCV or HBV RNA and methods for their use alone or in combination with other therapies. In addition, nucleic acid decoy molecules and aptamers that bind to HBV reverse transcriptase and/or HBV reverse transcriptase primer sequences and methods for their use alone or in combination with other therapies, are disclosed. Oligonucleotides that specifically bind the Enhancer I region of HBV DNA are further disclosed. The present invention further relates to the use of nucleic acids, such as decoy and aptamer molecules of the invention, to modulate the expression of Hepatitis B virus (HBV) genes and HBV viral replication. Furthermore, HBV animal models and methods of use are disclosed, including methods of screening for compounds and/or potential therapies directed against HBV. The present invention also relates to compounds, including enzymatic nucleic acid

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DESCRIPTION

OLIGONUCLEOTIDE MEDIATED INHIBITION OF HEPATITIS B VIRUS AND HEPATITIS C VIRUS REPLICATION

Background Of The Invention

This patent application claims priority from Blatt et al., USSN (09/817,879), filed March 26, 2001, which is a continuation-in-part of Blatt et al., USSN (09/740,332), filed December 18, 2000, which is a continuation-in-part of Blatt et al., USSN (09/611,931), filed July 7, 2000, which is a continuation-in-part of Blatt et al., 09/504,321, filed February 15, 2000, which is a continuation-in-part of Blatt et al., USSN 09/274,553, filed March 23, 1999, which is a continuation-in-part of Blatt et al., USSN 09/257,608, filed February 24, 1999 (abandoned), which claims priority from Blatt et al., USSN 60/100,842, filed September 18, 1998, and McSwiggen et al., USSN 60/083,217 filed April 27, 1998; all of these earlier applications are entitled "ENZYMATIC NUCLEIC ACID TREATMENT OF DISEASES OR CONDITIONS RELATED TO HEPATITIS C VIRUS INFECTION". This patent application also claims priority from Draper et al., USSN 09/877,478 filed June 8, 2001, which is a continuation-in-part of Draper et al., USSN (09/696,347), filed October 24, 2000, which is a continuation-in-part of Draper et al., USSN (09/636,385), filed August 9, 2000, which is a continuation in part of Draper et al., USSN (09/531,025), filed March 20, 2000, which is a continuation in part of Draper, USSN (09/436,430), filed November 8, 1999, which is a continuation of USSN (08/193,627), filed February 7, 1994, now US patent No. 6,017,756, which is a continuation of USSN (07/882,712), filed May 14, 1992, now abandoned; all of these earlier applications are entitled "METHOD AND REAGENT FOR INHIBITING HEPATITIS B VIRUS REPLICATION". This patent application also claims priority from Macejak et al., USSN (60/335,059), filed October 24, 2001, Macejak et al., USSN (60/296,876), filed June 8, 2001, and Morrissey et al., USSN (60/337,055), filed December 5, 2001. These applications are hereby incorporated by reference herein in their entireties, including the drawings.

The present invention concerns compounds, compositions, and methods for the study, diagnosis, and treatment of degenerative and disease states related to hepatitis B virus (HBV) and hepatitis C virus (HCV) infection, replication and gene expression. Specifically, the invention relates to nucleic acid molecules used to modulate expression of HBV and HCV. In

addition, the instant invention relates to methods, models and systems for screening inhibitors of HBV and HCV replication and propagation.

The following is a discussion of relevant art pertaining to hepatitis B virus (HBV) and hepatitis C virus (HCV). The discussion is not meant to be complete and is provided only for understanding of the invention that follows. The summary is not an admission that any of the work described below is prior art to the claimed invention.

In 1989, the Hepatitis C Virus (HCV) was determined to be an RNA virus and was identified as the causative agent of most non-A non-B viral Hepatitis (Choo *et al., Science.* 1989; 244:359-362). Unlike retroviruses such as HIV, HCV does not go though a DNA replication phase and no integrated forms of the viral genome into the host chromosome have been detected (Houghton *et al., Hepatology* 1991;14:381-388). Rather, replication of the coding (plus) strand is mediated by the production of a replicative (minus) strand leading to the generation of several copies of plus strand HCV RNA. The genome consists of a single, large, open-reading frame that is translated into a polyprotein (Kato *et al., FEBS Letters.* 1991; 280: 325-328). This polyprotein subsequently undergoes post-translational cleavage, producing several viral proteins (Leinbach *et al., Virology.* 1994: 204:163-169).

Examination of the 9.5-kilobase genome of HCV has demonstrated that the viral nucleic acid can mutate at a high rate (Smith *et al.,Mol. Evol.* 1997 45:238-246). This rate of mutation has led to the evolution of several distinct genotypes of HCV that share approximately 70% sequence identity (Simmonds *et al., J. Gen. Virol.* 1994;75 :1053-1061). It is important to note that these sequences are evolutionarily quite distant. For example, the genetic identity between humans and primates such as the chimpanzee is approximately 98%. In addition, it has been demonstrated that an HCV infection in an individual patient is composed of several distinct and evolving quasispecies that have 98% identity at the RNA level. Thus, the HCV genome is hypervariable and continuously changing. Although the HCV genome is hypervariable, there are 3 regions of the genome that are highly conserved. These conserved sequences occur in the 5' and 3' non-coding regions as well as the 5'-end of the core protein coding region and are thought to be vital for HCV RNA replication as well as translation of the HCV polyprotein. Thus, therapeutic agents that target these conserved HCV genomic regions can have a significant impact over a wide range of HCV genotypes. Moreover, it is unlikely that drug resistance will occur with enzymatic nucleic acids specific to conserved regions of the HCV genome. In contrast, therapeutic modalities that target inhibition of enzymes such as the viral proteases or helicase are likely to result in the selection for drug resistant strains since the RNA for these viral encoded enzymes is located in the hypervariable portion of the HCV genome.

After initial exposure to HCV, the patient experiences a transient rise in liver enzymes, which indicates the occurrence of inflammatory processes (Alter *et al.*, IN: Seeff LB, Lewis JH, eds. *Current Perspectives in Hepatology.* New York: Plenum Medical Book Co; 1989:83-89). This elevation in liver enzymes will occur at least 4 weeks after the initial exposure and can last for up to two months (Farci *et al., New England Journal of Medicine.* 1991:325:98-104). Prior to the rise in liver enzymes, it is possible to detect HCV RNA in the patient's serum using RT-PCR analysis (Takahashi *et al., American Journal of Gastroenterology.* 1993:88:2:240-243). This stage of the disease is called the acute stage and usually goes undetected since 75% of patients with acute viral hepatitis from HCV infection are asymptomatic. The remaining 25% of these patients develop jaundice or other symptoms of hepatitis.

Acute HCV infection is a benign disease, however, and as many as 80% of acute HCV patients progress to chronic liver disease as evidenced by persistent elevation of serum alanine aminotransferase (ALT) levels and by continual presence of circulating HCV RNA (Sherlock, *Lancet* 1992; 339:802). The natural progression of chronic HCV infection over a 10 to 20 year period leads to cirrhosis in 20 to 50% of patients (Davis *et al., Infectious Agents and Disease* 1993;2:150:154) and progression of HCV infection to hepatocellular carcinoma has been well documented (Liang *et al., Hepatology.* 1993; 18:1326-1333; Tong *et al., Western Journal of Medicine,* 1994; Vol. 160, No. 2: 133-138). There have been no studies that have determined sub-populations that are most likely to progress to cirrhosis and/or hepatocellular carcinoma, thus all patients have equal risk of progression.

It is important to note that the survival for patients diagnosed with hepatocellular carcinoma is only 0.9 to 12.8 months from initial diagnosis (Takahashi *et al., American Journal of Gastroenterology.* 1993:88:2:240-243). Treatment of hepatocellular carcinoma with chemotherapeutic agents has not proven effective and only 10% of patients will benefit from surgery due to extensive tumor invasion of the liver (Trinchet *et al., Presse Medicine.* 1994:23:831-833). Given the aggressive nature of primary hepatocellular carcinoma, the only viable treatment alternative to surgery is liver transplantation (Pichlmayr *et al., Hepatology.* 1994:20:33S-40S).

Upon progression to cirrhosis, patients with chronic HCV infection present with clinical features, which are common to clinical cirrhosis regardless of the initial cause (D'Amico *et al., Digestive Diseases and Sciences.* 1986;31:5: 468-475). These clinical features can include: bleeding esophageal varices, ascites, jaundice, and encephalopathy (Zakim D, Boyer TD. Hepatology a textbook of liver disease. Second Edition Volume 1. 1990 W.B. Saunders Company. Philadelphia). In the early stages of cirrhosis, patients are classified as compensated, meaning that although liver tissue damage has occurred, the patient's liver is still able to detoxify metabolites in the blood-stream. In addition, most

patients with compensated liver disease are asymptomatic and the minority with symptoms report only minor symptoms such as dyspepsia and weakness. In the later stages of cirrhosis, patients are classified as decompensated meaning that their ability to detoxify metabolites in the bloodstream is diminished and it is at this stage that the clinical features described above will present.

In 1986, D'Amico *et al.* described the clinical manifestations and survival rates in 1155 patients with both alcoholic and viral associated cirrhosis (D'Amico *supra*). Of the 1155 patients, 435 (37%) had compensated disease although 70% were asymptomatic at the beginning of the study. The remaining 720 patients (63%) had decompensated liver disease with 78% presenting with a history of ascites, 31% with jaundice, 17% had bleeding and 16% had encephalopathy. Hepatocellular carcinoma was observed in six (.5%) patients with compensated disease and in 30 (2.6%) patients with decompensated disease.

Over the course of six years, the patients with compensated cirrhosis developed clinical features of decompensated disease at a rate of 10% per year. In most cases, ascites was the first presentation of decompensation. In addition, hepatocellular carcinoma developed in 59 patients who initially presented with compensated disease by the end of the six-year study.

With respect to survival, the D'Amico study indicated that the five-year survival rate for all patients on the study was only 40%. The six-year survival rate for the patients who initially had compensated cirrhosis was 54%, while the six-year survival rate for patients who initially presented with decompensated disease was only 21%. There were no significant differences in the survival rates between the patients who had alcoholic cirrhosis and the patients with viral related cirrhosis. The major causes of death for the patients in the D'Amico study were liver failure in 49%; hepatocellular carcinoma in 22%; and, bleeding in 13% (D'Amico *supra*).

Chronic Hepatitis C is a slowly progressing inflammatory disease of the liver, mediated by a virus (HCV) that can lead to cirrhosis, liver failure and/or hepatocellular carcinoma over a period of 10 to 20 years. In the US, it is estimated that infection with HCV accounts for 50,000 new cases of acute hepatitis in the United States each year (NIH Consensus Development Conference Statement on Management of Hepatitis C March 1997). The prevalence of HCV in the United States is estimated at 1.8% and the CDC places the number of chronically infected Americans at approximately 4.5 million people. The CDC also estimates that up to 10,000 deaths per year are caused by chronic HCV infection. The prevalence of HCV in the United States is estimated at 1.8% and the CDC places the number of chronically infected Americans at approximately 4.5 million people. The CDC also estimates that up to 10,000 deaths per year are caused by chronic HCV infection.

Numerous well controlled clinical trials using interferon (IFN-alpha) in the treatment of chronic HCV infection have demonstrated that treatment three times a week results in lowering of serum ALT values in approximately 50% (range 40% to 70%) of patients by the end of 6 months of therapy (Davis *et al., New England Journal of Medicine* 1989; 321:1501-1506; Marcellin *et al., Hepatology.* 1991; 13:393-397; Tong *et al., Hepatology* 1997:26:747-754; Tong *et al., Hepatology* 1997 26(6): 1640-1645). However, following cessation of interferon treatment, approximately 50% of the responding patients relapsed, resulting in a "durable" response rate as assessed by normalization of serum ALT concentrations of approximately 20 to 25%.

In recent years, direct measurement of the HCV RNA has become possible through use of either the branched-DNA or Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) analysis. In general, the RT-PCR methodology is more sensitive and leads to more accurate assessment of the clinical course (Tong *et al., supra*). Studies that have examined six months of type 1 interferon therapy using changes in HCV RNA values as a clinical endpoint have demonstrated that up to 35% of patients will have a loss of HCV RNA by the end of therapy (Marcellin *et al., supra*). However, as with the ALT endpoint, about 50% of the patients relapse six months following cessation of therapy resulting in a durable virologic response of only 12% (Marcellin *et al., supra*). Studies that have examined 48 weeks of therapy have demonstrated that the sustained virological response is up to 25% (NIH consensus statement: 1997). Thus, standard of care for treatment of chronic HCV infection with type 1 interferon is now 48 weeks of therapy using changes in HCV RNA concentrations as the primary assessment of efficacy (Hoofnagle *et al., New England Journal of Medicine* 1997; 336(5) 347-356).

Side effects resulting from treatment with type 1 interferons can be divided into four general categories, which include 1. Influenza-like symptoms; 2. Neuropsychiatric; 3. Laboratory abnormalities; and, 4. Miscellaneous (Dusheiko *et al.,* Journal of Viral Hepatitis. 1994:1:3-5). Examples of influenza-like symptoms include; fatigue, fever; myalgia; malaise; appetite loss; tachycardia; rigors; headache and arthralgias. The influenza-like symptoms are usually short-lived and tend to abate after the first four weeks of dosing (Dushieko *et al., supra*). Neuropsychiatric side effects include: irritability, apathy; mood changes; insomnia; cognitive changes and depression. The most important of these neuropsychiatric side effects is depression and patients who have a history of depression should not be given type 1 interferon. Laboratory abnormalities include; reduction in myeloid cells including granulocytes, platelets and to a lesser extent red blood cells. These changes in blood cell counts rarely lead to any significant clinical sequellae (Dushieko *et al., supra*). In addition, increases in triglyceride concentrations and elevations in serum alanine and aspartate aminotransferase concentration have been observed. Finally, thyroid abnormalities have been reported. These thyroid abnormalities are usually reversible after cessation of interferon

therapy and can be controlled with appropriate medication while on therapy. Miscellaneous side effects include nausea; diarrhea; abdominal and back pain; pruritus; alopecia; and rhinorrhea. In general, most side effects will abate after 4 to 8 weeks of therapy (Dushieko *et al., supra*).

Type 1 Interferon is a key constituent of many treatment programs for chronic HCV infection. Treatment with type 1 interferon induces a number of genes and results in an antiviral state within the cell. One of the genes induced is 2', 5' oligoadenylate synthetase, an enzyme that synthesizes short 2', 5' oligoadenylate (2-5A) molecules. Nascent 2-5A subsequently activates a latent RNase, RNase L, which in turn nonspecifically degrades viral RNA.

Chronic hepatitis B is caused by an enveloped virus, commonly known as the hepatitis B virus or HBV. HBV is transmitted via infected blood or other body fluids, especially saliva and semen, during delivery, sexual activity, or sharing of needles contaminated by infected blood. Individuals may be "carriers" and transmit the infection to others without ever having experienced symptoms of the disease. Persons at highest risk are those with multiple sex partners, those with a history of sexually transmitted diseases, parenteral drug users, infants born to infected mothers, "close" contacts or sexual partners of infected persons, and healthcare personnel or other service employees who have contact with blood. Transmission is also possible via tattooing, ear or body piercing, and acupuncture; the virus is also stable on razors, toothbrushes, baby bottles, eating utensils, and some hospital equipment such as respirators, scopes and instruments. There is no evidence that HBsAg positive food handlers pose a health risk in an occupational setting, nor should they be excluded from work. Hepatitis B has never been documented as being a food-borne disease. The average incubation period is 60 to 90 days, with a range of 45 to 180; the number of days appears to be related to the amount of virus to which the person was exposed. However, determining the length of incubation is difficult, since onset of symptoms is insidious. Approximately 50% of patients develop symptoms of acute hepatitis that last from 1 to 4 weeks. Two percent or less of these individuals develop fulminant hepatitis resulting in liver failure and death.

The determinants of severity include: (1) The size of the dose to which the person was exposed; (2) the person's age with younger patients experiencing a milder form of the disease; (3) the status of the immune system with those who are immunosuppressed experiencing milder cases; and (4) the presence or absence of co-infection with the Delta virus (hepatitis D), with more severe cases resulting from co-infection. In symptomatic cases, clinical signs include loss of appetite, nausea, vomiting, abdominal pain in the right upper quadrant, arthralgia, and tiredness/loss of energy. Jaundice is not experienced in all

cases, however, jaundice is more likely to occur if the infection is due to transfusion or percutaneous serum transfer, and it is accompanied by mild pruritus in some patients. Bilirubin elevations are demonstrated in dark urine and clay-colored stools, and liver enlargement may occur accompanied by right upper-quadrant pain. The acute phase of the disease may be accompanied by severe depression, meningitis, Guillain-Barré syndrome, myelitis, encephalitis, agranulocytosis, and/or thrombocytopenia.

Hepatitis B is generally self-limiting and will resolve in approximately 6 months. Asymptomatic cases can be detected by serologic testing, since the presence of the virus leads to production of large amounts of HBsAg in the blood. This antigen is the first and most useful diagnostic marker for active infections. However, if HBsAg remains positive for 20 weeks or longer, the person is likely to remain positive indefinitely and is now a carrier. While only 10% of persons over age 6 who contract HBV become carriers, 90% of infants infected during the first year of life do so.

Hepatitis B virus (HBV) infects over 300 million people worldwide (Imperial, 1999, *Gastroenterol. Hepatol.*, 14 (suppl), S1-5). In the United States, approximately 1.25 million individuals are chronic carriers of HBV as evidenced by the fact that they have measurable hepatitis B virus surface antigen HBsAg in their blood. The risk of becoming a chronic HBsAg carrier is dependent upon the mode of acquisition of infection as well as the age of the individual at the time of infection. For those individuals with high levels of viral replication, chronic active hepatitis with progression to cirrhosis, liver failure and hepatocellular carcinoma (HCC) is common, and liver transplantation is the only treatment option for patients with end-stage liver disease from HBV.

The natural progression of chronic HBV infection over a 10 to 20 year period leads to cirrhosis in 20-to-50% of patients and progression of HBV infection to hepatocellular carcinoma has been well documented. There have been no studies that have determined sub-populations that are most likely to progress to cirrhosis and/or hepatocellular carcinoma, thus all patients have equal risk of progression.

It is important to note that the survival for patients diagnosed with hepatocellular carcinoma is only 0.9 to 12.8 months from initial diagnosis (Takahashi *et al.*, 1993, *American Journal of Gastroenterology*, 88, 240-243). Treatment of hepatocellular carcinoma with chemotherapeutic agents has not proven effective and only 10% of patients will benefit from surgery due to extensive tumor invasion of the liver (Trinchet *et al.*, 1994,*Presse Medicine*, 23, 831-833). Given the aggressive nature of primary hepatocellular carcinoma, the only viable treatment alternative to surgery is liver transplantation (Pichlmayr *et al.*, 1994, *Hepatology.*, 20, 33S-40S).

Upon progression to cirrhosis, patients with chronic HCV and HBV infection present with clinical features, which are common to clinical cirrhosis regardless of the initial cause (D'Amico *et al.*, 1986, *Digestive Diseases and Sciences*, 31, 468-475). These clinical features may include: bleeding esophageal varices, ascites, jaundice, and encephalopathy (Zakim D, Boyer TD. *Hepatology a textbook of liver disease*, Second Edition Volume 1. 1990 W.B. Saunders Company. Philadelphia). In the early stages of cirrhosis, patients are classified as compensated, meaning that although liver tissue damage has occurred, the patient's liver is still able to detoxify metabolites in the blood-stream. In addition, most patients with compensated liver disease are asymptomatic and the minority with symptoms report only minor symptoms such as dyspepsia and weakness. In the later stages of cirrhosis, patients are classified as decompensated meaning that their ability to detoxify metabolites in the bloodstream is diminished and it is at this stage that the clinical features described above will present.

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Hepatitis B virus is a double-stranded circular DNA virus. It is a member of the Hepadnaviridae family. The virus consists of a central core that contains a core antigen (HBcAg) surrounded by an envelope containing a surface protein/surface antigen (HBsAg)

and is 42 nm in diameter. It also contains an e antigen (HBeAg), which, along with HBcAg and HBsAg, is helpful in identifying this disease.

In HBV virions, the genome is found in an incomplete double-stranded form. HBV uses a reverse transcriptase to transcribe a positive-sense full length RNA version of its genome back into DNA. This reverse transcriptase also contains DNA polymerase activity and thus begins replicating the newly synthesized minus-sense DNA strand. However, it appears that the core protein encapsidates the reverse-transcriptase/polymerase before it completes replication.

From the free-floating form, the virus must first attach itself specifically to a host cell membrane. Viral attachment is one of the crucial steps that determines host and tissue specificity. However, currently there are no *in vitro* cell-lines that can be infected by HBV. There are some cells lines, such as HepG2, which can support viral replication only upon transient or stable transfection using HBV DNA.

After attachment, fusion of the viral envelope and host membrane must occur to allow the viral core proteins containing the genome and polymerase to enter the cell. Once inside, the genome is translocated to the nucleus where it is repaired and cyclized.

The complete closed circular DNA genome of HBV remains in the nucleus and gives rise to four transcripts. These transcripts initiate at unique sites but share the same 3'-ends. The 3.5-kb pregenomic RNA serves as a template for reverse transcription and also encodes the nucleocapsid protein and polymerase. A subclass of this transcript with a 5'-end extension codes for the precore protein that, after processing, is secreted as HBV e antigen. The 2.4-kb RNA encompasses the pre-S1 open reading frame (ORF) that encodes the large surface protein. The 2.1-kb RNA encompasses the pre-S2 and S ORFs that encode the middle and small surface proteins, respectively. The smallest transcript (~0.8-kb) codes for the X protein, a transcriptional activator.

Multiplication of the HBV genome begins within the nucleus of an infected cell. RNA polymerase II transcribes the circular HBV DNA into greater-than-full length mRNA. Since the mRNA is longer than the actual complete circular DNA, redundant ends are formed. Once produced, the pregenomic RNA exits the nucleus and enters the cytoplasm.

The packaging of pregenomic RNA into core particles is triggered by the binding of the HBV polymerase to the 5' epsilon stem-loop. RNA encapsidation is believed to occur as soon as binding occurs. The HBV polymerase also appears to require associated core protein in order to function. The HBV polymerase initiates reverse transcription from the 5' epsilon stem-loop three to four base pairs at which point the polymerase and attached nascent DNA

are transferred to the 3' copy of the DR1 region. Once there, the (-)DNA is extended by the HBV polymerase while the RNA template is degraded by the HBV polymerase RNAse H activity. When the HBV polymerase reaches the 5' end, a small stretch of RNA is left undigested by the RNAse H activity. This segment of RNA is comprised of a small sequence just upstream and including the DR1 region. The RNA oligomer is then translocated and annealed to the DR2 region at the 5' end of the (-)DNA. It is used as a primer for the (+)DNA synthesis which is also generated by the HBV polymerase. It appears that the reverse transcription as well as plus strand synthesis may occur in the completed core particle.

Since the pregenomic RNA is required as a template for DNA synthesis, this RNA is an excellent target for nucleic acid based therapeutics. Nucleoside analogues that have been documented to modulate HBV replication target the reverse transcriptase activity needed to convert the pregenomic RNA into DNA. Nucleic acid decoy and aptamer modulation of HBV reverse transcriptase would be expected to result in a similar modulation of HBV replication.

Current therapeutic goals of treatment are three-fold: to eliminate infectivity and transmission of HBV to others, to arrest the progression of liver disease and improve the clinical prognosis, and to prevent the development of hepatocellular carcinoma (HCC).

Interferon alpha use is the most common therapy for HBV; however, recently Lamivudine (3TC®) has been approved by the FDA. Interferon alpha (IFN-alpha) is one treatment for chronic hepatitis B. The standard duration of IFN-alpha therapy is 16 weeks, however, the optimal treatment length is still poorly defined. A complete response (HBV DNA negative HBeAg negative) occurs in approximately 25% of patients. Several factors have been identified that predict a favorable response to therapy including:, High ALT, low HBV DNA, being female, and heterosexual orientation.

There is also a risk of reactivation of the hepatitis B virus even after a successful response, this occurs in around 5% of responders and normally occurs within 1 year.

Side effects resulting from treatment with type 1 interferons can be divided into four general categories including: Influenza-like symptoms, neuropsychiatric, laboratory abnormalities, and other miscellaneous side effects. Examples of influenza-like symptoms include, fatigue, fever, myalgia, malaise, appetite loss, tachycardia, rigors, headache and arthralgias. The influenza-like symptoms are usually short-lived and tend to abate after the first four weeks of dosing (Dusheiko *et al.*, 1994, *Journal of Viral Hepatitis*, 1, 3-5). Neuropsychiatric side effects include irritability, apathy, mood changes, insomnia, cognitive

changes, and depression. Laboratory abnormalities include the reduction of myeloid cells, including granulocytes, platelets and to a lesser extent, red blood cells. These changes in blood cell counts rarely lead to any significant clinical sequellae. In addition, increases in triglyceride concentrations and elevations in serum alanine and aspartate aminotransferase concentration have been observed. Finally, thyroid abnormalities have been reported. These thyroid abnormalities are usually reversible after cessation of interferon therapy and can be controlled with appropriate medication while on therapy. Miscellaneous side effects include nausea, diarrhea, abdominal and back pain, pruritus, alopecia, and rhinorrhea. In general, most side effects will abate after 4 to 8 weeks of therapy (Dushieko *et al., supra*).

Lamivudine (3TC®) is a nucleoside analogue, which is a very potent and specific inhibitor of HBV DNA synthesis. Lamivudine has recently been approved for the treatment of chronic Hepatitis B. Unlike treatment with interferon, treatment with 3TC® does not eliminate the HBV from the patient. Rather, viral replication is controlled and chronic administration results in improvements in liver histology in over 50% of patients. Phase III studies with 3TC®, showed that treatment for one year was associated with reduced liver inflammation and a delay in scarring of the liver. In addition, patients treated with Lamivudine (100mg per day) had a 98 percent reduction in hepatitis B DNA and a significantly higher rate of seroconversion, suggesting disease improvements after completion of therapy. However, stopping of therapy resulted in a reactivation of HBV replication in most patients. In addition recent reports have documented 3TC® resistance in approximately 30% of patients.

Current therapies for treating HBV infection, including interferon and nucleoside analogues, are only partially effective. In addition, drug resistance to nucleoside analogues is now emerging, making treatment of chronic Hepatitis B more difficult. Thus, a need exists for effective treatment of this disease that utilizes antiviral modulators that work by mechanisms other than those currently utilized in the treatment of both acute and chronic hepatitis B infections.

Welch *et al., Gene Therapy* 1996 3(11): 994-1001 describe *in vitro* an *in vivo* studies with two vector expressed hairpin ribozymes targeted against hepatitis C virus.

Sakamoto *et al., J. Clinical Investigation* 1996 98(12): 2720-2728 describe intracellular cleavage of hepatitis C virus RNA and inhibition of viral protein translation by certain vector expressed hammerhead ribozymes.

Lieber *et al., J. Virology* 1996 70(12): 8782-8791 describe elimination of hepatitis C virus RNA in infected human hepatocytes by adenovirus-mediated expression of certain hammerhead ribozymes.

Ohkawa *et al.*, 1997, *J. Hepatology*, 27; 78-84, describe *in vitro* cleavage of HCV RNA and inhibition of viral protein translation using certain *in vitro* transcribed hammerhead ribozymes.

Barber *et al.*, International PCT Publication No. *WO 97/32018*, describe the use of an adenovirus vector to express certain anti-hepatitis C virus hairpin ribozymes.

Kay *et al.*, International PCT Publication No. *WO 96/18419*, describe certain recombinant adenovirus vectors to express anti-HCV hammerhead ribozymes.

Yamada *et al.*, Japanese Patent Application No. *JP 07231784* describe a specific poly-(L)-lysine conjugated hammerhead ribozyme targeted against HCV.

Draper, U.S. Patent Nos. 5,610,054 and 5,869,253, describes enzymatic nucleic acid molecules capable of inhibiting replication of HCV.

Macejak. *et al.*, 2000, *Hepatology*, 31, 769-776, describe enzymatic nucleic acid molecules capable of inhibiting replication of HCV.

Weifeng and Torrence, 1997, *Nucleosides and Nucleotides*, 16, 7-9, describe the synthesis of 2-5A antisense chimeras with various non-nucleoside components.

Torrence *et al.*, US patent No. 5,583,032 describe targeted cleavage of RNA using an antisense oligonulceotide linked to a 2',5'-oligoadenylate activator of RNase L.

Suhadolnik and Pfleiderer, US patent Nos. 5,863,905; 5,700,785; 5,643,889; 5,556,840; 5,550,111; 5,405,939; 5,188,897; 4,924,624; and 4,859,768 describe specific internucleotide phosphorothioate 2',5'-oligoadenlyates and 2',5'-oligoadenlyate conjugates.

Budowsky *et al.*, US patent No. 5,962,431 describe a method of treating papillomavirus using specific 2',5'-oligoadenylates.

Torrence *et al.*, International PCT publication No. WO 00/14219, describe specific peptide nucleic acid 2',5'-oligoadenylate chimeric molecules.

Stinchcomb *et al.*, US patent No. 5,817,796, describe C-myb ribozymes having 2'-5'-Linked Adenylate Residues.

Draper, US patent No. 6,017,756, describes the use of ribozymes for the inhibition of Hepatitis B Virus.

Passman *et al.*, 2000, *Biochem. Biophys. Res. Commun.*, 268(3), 728-733.; Gan *et al.*, 1998, *J. Med. Coll. PLA*, 13(3), 157-159.; Li *et al.*, 1999, *Jiefangjun Yixue Zazhi*, 24(2), 99-

101.; Putlitz *et al.*, 1999, *J. Virol.*, 73(7), 5381-5387.; Kim *et al.*, 1999, *Biochem. Biophys. Res. Commun.*, 257(3), 759-765.; Xu *et al.*, 1998, *Bingdu Xuebao*, 14(4), 365-369.; Welch *et al.*, 1997, *Gene Ther.*, 4(7), 736-743.; Goldenberg *et al.*, 1997, International PCT publication No. WO 97/08309, Wands *et al.*, 1997, *J. of Gastroenterology and Hepatology*, 12(suppl.), S354-S369.; Ruiz *et al.*, 1997, *BioTechniques*, 22(2), 338-345.; Gan *et al.*, 1996, *J. Med. Coll. PLA*, 11(3), 171-175.; Beck and Nassal, 1995, *Nucleic Acids Res.*, 23(24), 4954-62.; Goldenberg, 1995, International PCT publication No. WO 95/22600.; Xu *et al.*, 1993, *Bingdu Xuebao*, 9(4), 331-6.; Wang *et al.*, 1993, *Bingdu Xuebao*, 9(3), 278-80, all describe ribozymes that are targeted to cleave a specific HBV target site.

Hunt *et al.*, US patent No. 5,859,226, describes specific non-naturally occurring oligonucleotide decoys intended to inhibit the expression of MHC-II genes through binding of the RF-X transcription factor, that can inhibit the expression of certain HBV and CMV viral proteins.

Kao *et al.*, International PCT Publication No. WO 00/04141, describes linear single stranded nucleic acid molecules capable of specifically binding to viral polymerases and inhibiting the activity of the viral polymerase.

Lu, International PCT Publication No. WO 99/20641, describes specific triplex-forming oligonucleotides used in treating HBV infection.

SUMMARY OF THE INVENTION

This invention relates to enzymatic nucleic acid molecules that can disrupt the function of RNA species of hepatitis B virus (HBV), hepatitis C virus (HCV) and/or those RNA species encoded by HBV or HCV. In particular, applicant provides enzymatic nucleic acid molecules capable of specifically cleaving HBV RNA or HCV RNA and describes the selection and function thereof. Such enzymatic nucleic acid molecules can be used to treat diseases and disorders associated with HBV and HCV infection.

In one embodiment, the invention features an enzymatic nucleic acid molecule that specifically cleaves RNA derived from hepatitis B virus (HBV), wherein the enzymatic nucleic acid molecule comprises sequence defined as Seq. ID No. 10887.

In another embodiment, the invention features a composition comprising an enzymatic nucleic acid molecule of the invention and a pharmaceutically acceptable carrier.

In another embodiment, the invention features a mammalian cell, for example a human cell, comprising an enzymatic nucleic acid molecule contemplated by the invention.

In one embodiment, the invention features a method for the treatment of cirrhosis, liver failure or hepatocellular carcinoma comprising administering to a patient an enzymatic nucleic acid molecule of the invention under conditions suitable for the treatment.

In another embodiment, the invention features a method for the treatment of a patient having a condition associated with HBV and/or HCV infection, comprising contacting cells of said patient with an enzymatic nucleic acid molecule of the invention.

In another embodiment, the invention features a method for the treatment of a patient having a condition associated with HBV and/or HCV infection, comprising contacting cells of said patient with an enzymatic nucleic acid molecule of the invention and further comprising the use of one or more drug therapies, for example, type I interferon or 3TC® (lamivudine), under conditions suitable for said treatment. In another embodiment, the other therapy is administered simultaneously with or separately from the enzymatic nucleic acid molecule.

In another embodiment, the invention features a method for inhibiting HBV and/or HCV replication in a mammalian cell comprising administering to the cell an enzymatic nucleic acid molecule of the invention under conditions suitable for the inhibition.

In yet another embodiment, the invention features a method of cleaving a separate HBV and/or HCV RNA comprising contacting an enzymatic nucleic acid molecule of the invention with the separate RNA under conditions suitable for the cleavage of the separate RNA.

In one embodiment, cleavage by an enzymatic nucleic acid molecule of the invention is carried out in the presence of a divalent cation, for example Mg2+.

In another embodiment, the enzymatic nucleic acid molecule of the invention is chemically synthesized.

In another embodiment, the type I interferon contemplated by the invention is interferon alpha, interferon beta, polyethylene glycol interferon, polyethylene glycol interferon alpha 2a, polyethylene glycol interferon alpha 2b, polyethylene glycol consensus interferon.

In one embodiment, the invention features a composition comprising type I interferon and an enzymatic nucleic acid molecule of the invention and a pharmaceutically acceptable carrier.

In another embodiment, the invention features a method of administering to a cell, for example a mammalian cell or human cell, an enzymatic nucleic acid molecule of the

invention independently or in conjunction with other therapeutic compounds, such as type I interferon or 3TC® (lamivudine), comprising contacting the cell with the enzymatic nucleic acid molecule under conditions suitable for the administration.

In another embodiment, administration of an enzymatic nucleic acid molecule of the invention is in the presence of a delivery reagent, for example a lipid, cationic lipid, phospholipid, or liposome.

In another embodiment, the invention features novel nucleic acid-based techniques such as enzymatic nucleic acid molecules and antisense molecules and methods for their use to down regulate or inhibit the expression of HBV RNA and/or replication of HBV.

In another embodiment, the invention features novel nucleic acid-based techniques such as enzymatic nucleic acid molecules and antisense molecules and methods for their use to down regulate or inhibit the expression of HCV RNA and/or replication of HCV.

In one embodiment, the invention features the use of one or more of the enzymatic nucleic acid-based techniques to down-regulate or inhibit the expression of the genes encoding HBV and/or HCV viral proteins. Specifically, the invention features the use of enzymatic nucleic acid-based techniques to specifically down-regulate or inhibit the expression of the HBV and/or HCV viral genome.

In another embodiment, the invention features nucleic acid-based inhibitors (*e.g.*, enzymatic nucleic acid molecules (ribozymes), antisense nucleic acids, triplex DNA, decoys, siRNA, aptamers, and antisense nucleic acids containing RNA cleaving chemical groups) and methods for their use to down regulate or inhibit the expression of RNA (*e.g.*, HBV and/or HCV) capable of progression and/or maintenance of hepatitis, hepatocellular carcinoma, cirrhosis, and/or liver failure.

In one embodiment, nucleic acid molecules of the invention are used to treat HBV infected cells or an HBV infected patient wherein the HBV is resistant or the patient does not respond to treatment with 3TC® (Lamivudine), either alone or in combination with other therapies under conditions suitable for the treatment.

In yet another embodiment, the invention features the use of an enzymatic nucleic acid molecule, preferably in the hammerhead, NCH (Inozyme), G-cleaver, amberzyme, zinzyme, and/or DNAzyme motif, to inhibit the expression of HBV and/or HCV RNA.

The enzymatic nucleic acid molecules described herein exhibit a high degree of specificity for only the viral mRNA in infected cells. Nucleic acid molecules of the instant invention targeted to highly conserved sequence regions allow the treatment of many strains

of human HBV and/or HCV with a single compound. No treatment presently exists which specifically attacks expression of the viral gene(s) that are responsible for transformation of hepatocytes by HBV and/or HCV.

The enzymatic nucleic acid-based modulators of HBV and HCV expression are useful for the prevention of the diseases and conditions including HBV and HCV infection, hepatitis, cancer, cirrhosis, liver failure, and any other diseases or conditions that are related to the levels of HBV and/or HCV in a cell or tissue.

Preferred target sites are genes required for viral replication, a non-limiting example includes genes for protein synthesis, such as the 5' most 1500 nucleotides of the HBV pregenomic mRNAs. For sequence references, see Renbao *et al.*, 1987, *Sci. Sin.*, 30, 507. This region controls the translational expression of the core protein (C), X protein (X) and DNA polymerase (P) genes and plays a role in the replication of the viral DNA by serving as a template for reverse transcriptase. Disruption of this region in the RNA results in deficient protein synthesis as well as incomplete DNA synthesis (and inhibition of transcription from the defective genomes). Targeting sequences 5' of the encapsidation site can result in the inclusion of the disrupted 3' RNA within the core virion structure and targeting sequences 3' of the encapsidation site can result in the reduction in protein expression from both the 3' and 5' fragments.

Alternative regions outside of the 5' most 1500 nucleotides of the pregenomic mRNA also make suitable targets for enzymatic nucleic acid mediated inhibition of HBV replication. Such targets include the mRNA regions that encode the viral S gene. Selection of particular target regions will depend upon the secondary structure of the pregenomic mRNA. Targets in the minor mRNAs can also be used, especially when folding or accessibility assays in these other RNAs reveal additional target sequences that are unavailable in the pregenomic mRNA species.

A desirable target in the pregenomic RNA is a proposed bipartite stem-loop structure in the 3'-end of the pregenomic RNA which is believed to be critical for viral replication (Kidd and Kidd-Ljunggren, 1996. *Nuc. Acid Res.* 24:3295-3302). The 5'end of the HBV pregenomic RNA carries a *cis*-acting encapsidation signal, which has inverted repeat sequences that are thought to form a bipartite stem-loop structure. Due to a terminal redundancy in the pregenomic RNA, the putative stem-loop also occurs at the 3'-end. While it is the 5' copy which functions in polymerase binding and encapsidation, reverse transcription actually begins from the 3' stem-loop. To start reverse transcription, a 4 nt primer which is covalently attached to the polymerase is made, using a bulge in the 5' encapsidation signal as template. This primer is then shifted, by an unknown mechanism, to the DR1 primer binding site in the 3' stem-loop structure, and reverse transcription proceeds

from that point. The 3' stem-loop, and especially the DR1 primer binding site, appear to be highly effective targets for ribozyme intervention.

Sequences of the pregenomic RNA are shared by the mRNAs for surface, core, polymerase, and X proteins. Due to the overlapping nature of the HBV transcripts, all share a common 3'-end. Enzymatic nucleic acids targeting of this common 3'-end will thus cleave the pregenomic RNA as well as all of the mRNAs for surface, core, polymerase and X proteins.

At least seven basic varieties of naturally-occurring enzymatic RNAs are known presently. Each can catalyze the hydrolysis of RNA phosphodiester bonds in *trans* (and thus can cleave other RNA molecules) under physiological conditions. **Table I** summarizes some of the characteristics of these enzymatic RNA molecules. In general, enzymatic nucleic acids act by first binding to a target RNA. Such binding occurs through the target binding portion of a enzymatic nucleic acid which is held in close proximity to an enzymatic portion of the molecule that acts to cleave the target RNA. Thus, the enzymatic nucleic acid first recognizes and then binds a target RNA through complementary base-pairing, and once bound to the correct site, acts enzymatically to cut the target RNA. Strategic cleavage of such a target RNA will destroy its ability to direct synthesis of an encoded protein. After an enzymatic nucleic acid has bound and cleaved its RNA target, it is released from that RNA to search for another target and can repeatedly bind and cleave new targets. Thus, a single enzymatic nucleic acid molecule is able to cleave many molecules of target RNA. In addition, the enzymatic nucleic acid is a highly specific inhibitor of gene expression, with the specificity of inhibition depending not only on the base-pairing mechanism of binding to the target RNA, but also on the mechanism of target RNA cleavage. Single mismatches, or base-substitutions, near the site of cleavage can completely eliminate catalytic activity of a an enzymatic nucleic acid molecule.

The enzymatic nucleic acid molecules that cleave the specified sites in HBV-specific RNAs represent a novel therapeutic approach to treat a variety of pathologic indications, including, HBV infection, hepatitis, hepatocellular carcinoma, tumorigenesis, cirrhosis, liver failure and other conditions related to the level of HBV.

In one of the preferred embodiments of the inventions described herein, the enzymatic nucleic acid molecule is formed in a hammerhead or hairpin motif, but can also be formed in the motif of a hepatitis delta virus, group I intron, group II intron or RNase P RNA (in association with an RNA guide sequence), *Neurospora* VS RNA, DNAzymes, NCH cleaving motifs, or G-cleavers. Examples of such hammerhead motifs are described by Dreyfus, *supra,* Rossi *et al.*, 1992, *AIDS Research and Human Retroviruses* 8, 183. Examples of hairpin motifs are described by Hampel *et al.*, EP0360257, Hampel and Tritz, 1989

Biochemistry 28, 4929, Feldstein *et al.*, 1989, *Gene* 82, 53, Haseloff and Gerlach, 1989, *Gene*, 82, 43, Hampel *et al.*, 1990 *Nucleic Acids Res.* 18, 299; and Chowrira & McSwiggen, US. Patent No. 5,631,359. The hepatitis delta virus motif is described by Perrotta and Been, 1992 *Biochemistry* 31, 16. The RNase P motif is described by Guerrier-Takada *et al.*, 1983 *Cell* 35, 849; Forster and Altman, 1990, *Science* 249, 783; and Li and Altman, 1996, *Nucleic Acids Res.* 24, 835. The *Neurospora* VS RNA ribozyme motif is described by Collins (Saville and Collins, 1990 *Cell* 61, 685-696; Saville and Collins, 1991 *Proc. Natl. Acad. Sci. USA* 88, 8826-8830; Collins and Olive, 1993 *Biochemistry* 32, 2795-2799; and Guo and Collins, 1995, *EMBO. J.* 14, 363). Group II introns are described by Griffin *et al.*, 1995, *Chem. Biol.* 2, 761; Michels and Pyle, 1995, *Biochemistry* 34, 2965; and Pyle *et al.*, International PCT Publication No. WO 96/22689. The Group I intron is described by Cech *et al.*, U.S. Patent 4,987,071. DNAzymes are described by Usman *et al.*, International PCT Publication No. WO 95/11304; Chartrand *et al.*, 1995, *NAR* 23, 4092; Breaker *et al.*, 1995, *Chem. Bio.* 2, 655; and Santoro *et al.*, 1997, *PNAS* 94, 4262. NCH cleaving motifs are described in Ludwig & Sproat, International PCT Publication No. WO 98/58058; and G-cleavers are described in Kore *et al.*, 1998, *Nucleic Acids Research* 26, 4116-4120 and Eckstein *et al.*, International PCT Publication No. WO 99/16871. Additional motifs include the Aptazyme (Breaker *et al.*, WO 98/43993), Amberzyme (Class I motif; **Figure 3**; Beigelman *et al.*, International PCT publication No. WO 99/55857) and Zinzyme (Beigelman *et al.*, International PCT publication No. WO 99/55857), all these references are incorporated by reference herein in their totalities, including drawings and can also be used in the present invention. These specific motifs are not limiting in the invention and those skilled in the art will recognize that all that is important in an enzymatic nucleic acid molecule of this invention is that it has a specific substrate binding site which is complementary to one or more of the target gene RNA regions, and that it have nucleotide sequences within or surrounding that substrate binding site which impart an RNA cleaving activity to the molecule (Cech *et al.*, U.S. Patent No. 4,987,071).

In preferred embodiments of the present invention, a nucleic acid molecule, *e.g.*, an antisense molecule, a triplex DNA, or a ribozyme, is 13 to 100 nucleotides in length, *e.g.*, in specific embodiments 35, 36, 37, or 38 nucleotides in length (*e.g.*, for particular ribozymes or antisense). In particular embodiments, the nucleic acid molecule is 15-100, 17-100, 20-100, 21-100, 23-100, 25-100, 27-100, 30-100, 32-100, 35-100, 40-100, 50-100, 60-100, 70-100, or 80-100 nucleotides in length. Instead of 100 nucleotides being the upper limit on the length ranges specified above, the upper limit of the length range can be, for example, 30, 40, 50, 60, 70, or 80 nucleotides. Thus, for any of the length ranges, the length range for particular embodiments has lower limit as specified, with an upper limit as specified which is greater than the lower limit. For example, in a particular embodiment, the length range can be 35-50 nucleotides in length. All such ranges are expressly included. Also in particular

embodiments, a nucleic acid molecule can have a length which is any of the lengths specified above, for example, 21 nucleotides in length.

Exemplary enzymatic nucleic acid molecules of the invention targeting HBV are shown in **Tables V-XI.** For example, enzymatic nucleic acid molecules of the invention are preferably between 15 and 50 nucleotides in length, more preferably between 25 and 40 nucleotides in length, *e.g.*, 34, 36, or 38 nucleotides in length (for example see Jarvis *et al.*, 1996, *J. Biol. Chem.*, 271, 29107-29112). Exemplary DNAzymes of the invention are preferably between 15 and 40 nucleotides in length, more preferably between 25 and 35 nucleotides in length, *e.g.*, 29, 30, 31, or 32 nucleotides in length (see for example Santoro *et al.*, 1998, *Biochemistry,* 37, 13330-13342; Chartrand *et al.*, 1995, Nucleic Acids Research, 23, 4092-4096). Exemplary antisense molecules of the invention are preferably between 15 and 75 nucleotides in length, more preferably between 20 and 35 nucleotides in length, *e.g.*, 25, 26, 27, or 28 nucleotides in length (see for example Woolf *et al.*, 1992, *PNAS.*, 89, 7305-7309; Milner *et al.*, 1997, *Nature Biotechnology,* 15, 537-541). Exemplary triplex forming oligonucleotide molecules of the invention are preferably between 10 and 40 nucleotides in length, more preferably between 12 and 25 nucleotides in length, *e.g.*, 18, 19, 20, or 21 nucleotides in length (see for example Maher *et al.*, 1990, *Biochemistry,* 29, 8820-8826; Strobel and Dervan, 1990, *Science,* 249, 73-75). Those skilled in the art will recognize that all that is required is for the nucleic acid molecule are of length and conformation sufficient and suitable for the nucleic acid molecule to catalyze a reaction contemplated herein. The length of the nucleic acid molecules of the instant invention are not limiting within the general limits stated.

In a preferred embodiment, the invention provides a method for producing a class of nucleic acid–based gene inhibiting agents which exhibit a high degree of specificity for the RNA of a desired target. For example, the enzymatic nucleic acid molecule is preferably targeted to a highly conserved sequence region of target RNAs encoding HBV proteins (specifically HBV RNA) such that specific treatment of a disease or condition can be provided with either one or several nucleic acid molecules of the invention. Such nucleic acid molecules can be delivered exogenously to specific tissue or cellular targets as required. Alternatively, the nucleic acid molecules (*e.g.*, ribozymes and antisense) can be expressed from DNA and/or RNA vectors that are delivered to specific cells.

The enzymatic nucleic acid-based inhibitors of HBV expression are useful for the prevention of the diseases and conditions including HBV infection, hepatitis, cancer, cirrhosis, liver failure, and any other diseases or conditions that are related to the levels of HBV in a cell or tissue.

The nucleic acid-based inhibitors of the invention are added directly, or can be complexed with cationic lipids, packaged within liposomes, or otherwise delivered to target cells or tissues. The nucleic acid or nucleic acid complexes can be locally administered to relevant tissues *ex vivo*, or *in vivo* through injection, infusion pump or stent, with or without their incorporation in biopolymers. In preferred embodiments, the enzymatic nucleic acid HBV inhibitors comprise sequences, which are complementary to the substrate sequences in. Examples of such enzymatic nucleic acid molecules also are shown in. Examples of such enzymatic nucleic acid molecules consist essentially of sequences defined in these tables.

In yet another embodiment, the invention features antisense nucleic acid molecules including sequences complementary to the HBV substrate sequences shown in. Such nucleic acid molecules can include sequences as shown for the binding arms of the enzymatic nucleic acid molecules in. Similarly, triplex molecules can be provided targeted to the corresponding DNA target regions, and regions containing the DNA equivalent of a target sequence or a sequence complementary to the specified target (substrate) sequence. Typically, antisense molecules are complementary to a target sequence along a single contiguous sequence of the antisense molecule. However, in certain embodiments, an antisense molecule can bind to substrate such that the substrate molecule forms a loop, and/or an antisense molecule can bind such that the antisense molecule forms a loop. Thus, the antisense molecule can be complementary to two (or even more) non-contiguous substrate sequences or two (or even more) non-contiguous sequence portions of an antisense molecule can be complementary to a target sequence or both.

By "consists essentially of" is meant that the active nucleic acid molecule of the invention, for example, an enzymatic nucleic acid molecule, contains an enzymatic center or core equivalent to those in the examples, and binding arms able to bind RNA such that cleavage at the target site occurs. Other sequences can be present which do not interfere with such cleavage. Thus, a core region can, for example, include one or more loops, stem-loop structure, or linker which does not prevent enzymatic activity. Thus, the underlined regions in the sequences in can be such a loop, stem-loop, nucleotide linker, and/or non-nucleotide linker and can be represented generally as sequence "X". For example, a core sequence for a hammerhead enzymatic nucleic acid can comprise a conserved sequence, such as 5'-CUGAUGAG-3' and 5'-CGAA-3' connected by "X", where X is 5'-GCCGUUAGGC-3' (SEQ ID NO. 16201), or any other Stem II region known in the art, or a nucleotide and/or non-nucleotide linker. Similarly, for other nucleic acid molecules of the instant invention, such as Inozyme, G-cleaver, amberzyme, zinzyme, DNAzyme, antisense, 2-5A antisense, triplex forming nucleic acid, and decoy nucleic acids, other sequences or non-nucleotide linkers can be present that do not interfere with the function of the nucleic acid molecule.

In another aspect of the invention, enzymatic nucleic acids or antisense molecules that interact with target RNA molecules and inhibit HBV (specifically HBV RNA) activity are expressed from transcription units inserted into DNA or RNA vectors. The recombinant vectors are preferably DNA plasmids or viral vectors. Enzymatic nucleic acid or antisense expressing viral vectors can be constructed based on, but not limited to, adeno-associated virus, retrovirus, adenovirus, or alphavirus. Preferably, the recombinant vectors capable of expressing the enzymatic nucleic acids or antisense are delivered as described above, and persist in target cells. Alternatively, viral vectors can be used that provide for transient expression of enzymatic nucleic acids or antisense. Such vectors can be repeatedly administered as necessary. Once expressed, the enzymatic nucleic acids or antisense bind to the target RNA and inhibit its function or expression. Delivery of enzymatic nucleic acids or antisense expressing vectors can be systemic, such as by intravenous or intramuscular administration, by administration to target cells ex-planted from the patient followed by reintroduction into the patient, or by any other means that allow for introduction into the desired target cell. Antisense DNA can be expressed via the use of a single stranded DNA intracellular expression vector.

In another embodiment, the invention features nucleic acid-based inhibitors (*e.g.*, enzymatic nucleic acid molecules (ribozymes), antisense nucleic acids, triplex DNA, decoys, aptamers, siRNA, antisense nucleic acids containing RNA cleaving chemical groups) and methods for their use to down regulate or inhibit the expression of RNA (*e.g.*, HBV) capable of progression and/or maintenance of liver disease and failure.

In another embodiment, the invention features nucleic acid-based techniques (*e.g.*, enzymatic nucleic acid molecules (ribozymes), antisense nucleic acids, triplex DNA, decoys, aptamers, siRNA, antisense nucleic acids containing RNA cleaving chemical groups) and methods for their use to down regulate or inhibit the expression of HBV RNA expression.

In other embodiments, the invention features a method for the analysis of HBV proteins. This method is useful in determining the efficacy of HBV inhibitors. Specifically, the instant invention features an assay for the analysis of HBsAg proteins and secreted alkaline phosphatase (SEAP) control proteins to determine the efficacy of agents used to modulate HBV expression.

The method consists of coating a micro-titer plate with an antibody such as anti-HBsAg Mab (for example, Biostride B88-95-31ad,ay) at 0.1 to 10 μg/ml in a buffer (for example, carbonate buffer, such as Na_2CO_3 15 mM, $NaHCO_3$ 35 mM, pH 9.5) at 4°C overnight. The microtiter wells are then washed with PBST or the equivalent thereof, (for example, PBS, 0.05% Tween 20) and blocked for 0.1-24 hr at 37° C with PBST, 1% BSA or the equivalent

Biotinylated goat anti-HBsAg or an equivalent antibody (for example, Accurate YVS1807) is diluted (for example at 1:1000) in PBST and incubated in the wells (for example, 1 hr. at 37° C). The wells are washed with PBST (for example, 4x). A conjugate, (for example, Streptavidin/Alkaline Phosphatase Conjugate, Pierce 21324) is diluted to 10-10,000 ng/ml in PBST, and incubated in the wells (for example, 1 hr. at 37° C). After washing as above, a substrate (for example, p-nitrophenyl phosphate substrate, Pierce 37620) is added to the wells, which are then incubated (for example, 1 hr. at 37° C). The optical density is then determined (for example, at 405 nm). SEAP levels are then assayed, for example, using the Great EscAPe® Detection Kit (Clontech K2041-1), as per the manufacturers instructions. In the above example, incubation times and reagent concentrations can be varied to achieve optimum results, a non-limiting example is described in Example 6.

Comparison of this HBsAg ELISA method to a commercially available assay from World Diagnostics, Inc. 15271 NW 60th Ave, #201, Miami Lakes, FL 33014 (305) 827-3304 (Cat. No. EL10018) demonstrates an increase in sensitivity (signal:noise) of 3-20 fold.

This invention also relates to nucleic acid molecules directed to disrupt the function of HBV reverse transcriptase. In addition, the invention relates to nucleic acid molecules directed to disrupt the function of the Enhancer I core region of the HBV genomic DNA. In particular, the present invention describes the selection and function of nucleic acid molecules, such as decoys and aptamers, capable of specifically binding to the HBV reverse transcriptase (pol) primer and modulating reverse transcription of the HBV pregenomic RNA. In another embodiment, the present invention relates to nucleic acid molecules, such as decoys, antisense and aptamers, capable of specifically binding to the HBV reverse transcriptase (pol) and modulating reverse transcription of the HBV pregenomic RNA. In yet another embodiment, the present invention relates to nucleic acid molecules capable of specifically binding to the HBV Enhancer I core region and modulating transcription of the HBV genomic DNA. The invention further relates to allosteric enzymatic nucleic acid molecules or "allozymes" that are used to modulate HBV gene expression. Such allozymes are active in the presence of HBV-derived nucleic acids, peptides, and/or proteins such as HBV reverse transcriptase and/or a HBV reverse transcriptase primer sequence, thereby allowing the allozyme to selectively cleave a sequence of HBV DNA or RNA. Allozymes of the invention are also designed to be active in the presence of HBV Enhancer I sequences and/or mutant HBV Enhancer I sequences, thereby allowing the allozyme to selectively cleave a sequence of HBV DNA or RNA. These nucleic acid molecules can be used to treat diseases and disorders associated with HBV infection.

In one embodiment, the invention features a nucleic acid decoy molecule that specifically binds the hepatitis B virus (HBV) reverse transcriptase primer sequence. In

another embodiment, the invention features a nucleic acid decoy molecule that specifically binds the hepatitis B virus (HBV) reverse transcriptase. In yet another embodiment, the invention features a nucleic acid decoy molecule that specifically binds to the HBV Enhancer I core sequence.

In one embodiment, the invention features a nucleic acid aptamer that specifically binds the hepatitis B virus (HBV) reverse transcriptase primer. In another embodiment, the invention features a nucleic acid aptamer that specifically binds the hepatitis B virus (HBV) reverse transcriptase. In yet another embodiment, the invention features a nucleic acid aptamer molecule that specifically binds to the HBV Enhancer I core sequence.

In one embodiment, the invention features an allozyme that specifically binds the hepatitis B virus (HBV) reverse transcriptase primer. In another embodiment, the invention features an allozyme that specifically binds the hepatitis B virus (HBV) reverse transcriptase. In yet another embodiment, the invention features an allozyme that specifically binds to the HBV Enhancer I core sequence.

In yet another embodiment, the invention features a nucleic acid molecule, for example a triplex forming nucleic acid molecule or antisense nucleic acid molecule, that binds the hepatitis B virus (HBV) reverse transcriptase primer. In another embodiment, the invention features a triplex forming nucleic acid molecule or antisense nucleic acid molecule that specifically binds the hepatitis B virus (HBV) reverse transcriptase. In yet another embodiment, the invention features a triplex forming nucleic acid molecule or antisense nucleic acid molecule that specifically binds to the HBV Enhancer I core sequence.

In another embodiment, a nucleic acid molecule of the invention binds to Hepatocyte Nuclear Factor 3 (HNF3) and/or Hepatocyte Nuclear Factor 4 (HNF4) binding sequence within the HBV Enhancer I region of HBV genomic DNA, for example the plus strand and/or minus strand DNA of the Enhancer I region, and blocks the binding of HNF3 and/or HNF4 to the Enhancer 1 region.

In another embodiment, the nucleic acid molecule of the invention comprises a sequence having $(UUCA)_n$ domain, where n is an integer from 1-10. In another embodiment, the nucleic acid molecules of the invention comprise the sequence of SEQ. ID NOs: 11216 - 11342.

In another embodiment, the invention features a composition comprising a nucleic acid molecule of the invention and a pharmaceutically acceptable carrier. In another embodiment, the invention features a mammalian cell, for example a human cell, including a nucleic acid molecule contemplated by the invention.

In one embodiment, the invention features a method for treatment of HBV infection, cirrhosis, liver failure, or hepatocellular carcinoma, comprising administering to a patient a nucleic acid molecule of the invention under conditions suitable for the treatment.

In another embodiment, the invention features a method for the treatment of a patient having a condition associated with HBV infection comprising contacting cells of said patient with a nucleic acid molecule of the invention under conditions suitable for such treatment. In another embodiment, the invention features a method for the treatment of a patient having a condition associated with HBV infection comprising contacting cells of said patient with a nucleic acid molecule of the invention, and further comprising the use of one or more drug therapies, for example type I interferon or 3TC® (lamivudine), under conditions suitable for said treatment. In another embodiment, the other therapy is administered simultaneously with or separately from the nucleic acid molecule.

In another embodiment, the invention features a method for modulating HBV replication in a mammalian cell comprising administering to the cell a nucleic acid molecule of the invention under conditions suitable for the modulation.

In yet another embodiment, the invention features a method of modulating HBV reverse transcriptase activity comprising contacting a nucleic acid molecule of the invention, for example a decoy or aptamer, with HBV reverse transcriptase under conditions suitable for the modulating of the HBV reverse transcriptase activity.

In another embodiment, the invention features a method of modulating HBV transcription comprising contacting a nucleic molecule of the invention with a HBV Enhancer I sequence under conditions suitable for the modulation of HBV transcription.

In one embodiment, a nucleic acid molecule of the invention, for example a decoy or aptamer, is chemically synthesized. In another embodiment, the nucleic acid molecule of the invention comprises at least one nucleic acid sugar modification. In yet another embodiment, the nucleic acid molecule of the invention comprises at least one nucleic acid base modification. In another embodiment, the nucleic acid molecule of the invention comprises at least one nucleic acid backbone modification.

In another embodiment, the nucleic acid molecule of the invention comprises at least one 2'-O-alkyl, 2'-alkyl, 2'-alkoxylalkyl, 2'-alkylthioalkyl, 2'-amino, 2'-O-amino, or 2'-halo modification and/or any combination thereof with or without 2'-deoxy and/or 2'-ribo nucleotides. In yet another embodiment, the nucleic acid molecule of the invention comprises all 2'-O-alkyl nucleotides, for example, all 2'-O-allyl nucleotides.

In one embodiment, the nucleic acid molecule of the invention comprises a 5'-cap, 3'-cap, or 5'-3' cap structure, for example an abasic or inverted abasic moiety.

In another embodiment, the nucleic acid molecule of the invention is a linear nucleic acid molecule. In another embodiment, the nucleic acid molecule of the invention is a linear nucleic acid molecule that can optionally form a hairpin, loop, stem-loop, or other secondary structure. In yet another embodiment, the nucleic acid molecule of the invention is a circular nucleic acid molecule.

In one embodiment, the nucleic acid molecule of the invention is a single stranded oligonucleotide. In another embodiment, the nucleic acid molecule of the invention is a double-stranded oligonucleotide.

In one embodiment, the nucleic acid molecule of the invention comprises an oligonucleotide having between about 3 and about 100 nucleotides. In another embodiment, the nucleic acid molecule of the invention comprises an oligonucleotide having between about 3 and about 24 nucleotides. In another embodiment, the nucleic acid molecule of the invention comprises an oligonucleotide having between about 4 and about 16 nucleotides.

The nucleic acid decoy molecules and/or aptamers that bind to a reverse transcriptase and/or reverse transcriptase primer and therefore inactivate the reverse transcriptase, represent a novel therapeutic approach to treat a variety of pathologic indications, including, viral infection such as HBV infection, hepatitis, hepatocellular carcinoma, tumorigenesis, cirrhosis, liver failure and others.

The nucleic acid molecules that bind to a HBV Enhancer I sequence and therefore inactivate HBV transcription, represent a novel therapeutic approach to treat a variety of pathologic indications, including viral infection such as HBV infection, hepatitis, hepatocellular carcinoma, tumorigenesis, cirrhosis, liver failure and others conditions associated with the level of HBV.

In one embodiment of the present invention, a decoy nucleic acid molecule of the invention is 4 to 50 nucleotides in length, in specific embodiments about 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or 16 nucleotides in length. In another embodiment, a non-decoy nucleic acid molecule, *e.g.*, an antisense molecule, a triplex DNA, or a ribozyme, is 13 to 100 nucleotides in length, *e.g.*, in specific embodiments 35, 36, 37, or 38 nucleotides in length (*e.g.*, for particular ribozymes or antisense). In particular embodiments, the nucleic acid molecule is 15-100, 17-100, 20-100, 21-100, 23-100, 25-100, 27-100, 30-100, 32-100, 35-100, 40-100, 50-100, 60-100, 70-100, or 80-100 nucleotides in length. Instead of 100 nucleotides being the upper limit on the length ranges specified above, the upper limit of the

length range can be, for example, 30, 40, 50, 60, 70, or 80 nucleotides. Thus, for any of the length ranges, the length range for particular embodiments has lower limit as specified, with an upper limit as specified which is greater than the lower limit. For example, in a particular embodiment, the length range can be 35-50 nucleotides in length. All such ranges are expressly included. Also in particular embodiments, a nucleic acid molecule can have a length which is any of the lengths specified above, for example, 21 nucleotides in length.

Exemplary nucleic acid decoy molecules of the invention are shown in **Table XIV**. Exemplary synthetic nucleic acid molecules of the invention are shown in **Table XV**. For example, decoy molecules of the invention are between 4 and 40 nucleotides in length. Exemplary decoys of the invention are 4, 8, 12, or 16 nucleotides in length. In an additional example, enzymatic nucleic acid molecules of the invention are preferably between 15 and 50 nucleotides in length, more preferably between 25 and 40 nucleotides in length, *e.g.*, 34, 36, or 38 nucleotides in length (for example see Jarvis *et al.*, 1996, *J. Biol. Chem.*, 271, 29107-29112). Exemplary DNAzymes of the invention are preferably between 15 and 40 nucleotides in length, more preferably between 25 and 35 nucleotides in length, *e.g.*, 29, 30, 31, or 32 nucleotides in length (see for example Santoro *et al.*, 1998, *Biochemistry*, 37, 13330-13342; Chartrand *et al.*, 1995, Nucleic Acids Research, 23, 4092-4096). Exemplary antisense molecules of the invention are preferably between 15 and 75 nucleotides in length, more preferably between 20 and 35 nucleotides in length, *e.g.*, 25, 26, 27, or 28 nucleotides in length (see for example Woolf *et al.*, 1992, *PNAS.*, 89, 7305-7309; Milner *et al.*, 1997, *Nature Biotechnology*, 15, 537-541). Exemplary triplex forming oligonucleotide molecules of the invention are preferably between 10 and 40 nucleotides in length, more preferably between 12 and 25 nucleotides in length, *e.g.*, 18, 19, 20, or 21 nucleotides in length (see for example Maher *et al.*, 1990, *Biochemistry*, 29, 8820-8826; Strobel and Dervan, 1990, *Science*, 249, 73-75). Those skilled in the art will recognize that all that is required is that the nucleic acid molecule is of length and conformation sufficient and suitable for the nucleic acid molecule to catalyze a reaction contemplated herein. The length of the nucleic acid molecules of the instant invention are not limiting within the general limits stated.

In one embodiment, the invention provides a method for producing a class of nucleic acid–based gene modulating agents, which exhibit a high degree of specificity for a viral reverse transcriptase such as HBV reverse transcriptase or reverse transcriptase primer such as a HBV reverse transcriptase primer. For example, the nucleic acid molecule is preferably targeted to a highly conserved nucleic acid binding region of the viral reverse transcriptase such that specific treatment of a disease or condition can be provided with either one or several nucleic acid molecules of the invention. Such nucleic acid molecules can be delivered exogenously to specific tissue or cellular targets as required. Alternatively, the

nucleic acid molecules can be expressed from DNA and/or RNA vectors that are delivered to specific cells.

In another embodiment, the invention provides a method for producing a class of nucleic acid–based gene modulating agents which exhibit a high degree of specificity for a viral enhancer regions such as the HBV Enhancer I core sequence. For example, the nucleic acid molecule is preferably targeted to a highly conserved transcription factor-binding region of the viral Enhancer I sequence such that specific treatment of a disease or condition can be provided with either one or several nucleic acid molecules of the invention. Such nucleic acid molecules can be delivered exogenously to specific tissue or cellular targets as required. Alternatively, the nucleic acid molecules can be expressed from DNA and/or RNA vectors that are delivered to specific cells.

In a another embodiment the invention provides a method for producing a class of enzymatic cleaving agents which exhibit a high degree of specificity for the RNA of a desired target. The enzymatic nucleic acid molecule, nuclease activating compound or chimera is preferably targeted to a highly conserved sequence region of a target mRNAs encoding HCV or HBV proteins such that specific treatment of a disease or condition can be provided with either one or several enzymatic nucleic acids. Such nucleic acid molecules can be delivered exogenously to specific cells as required. Alternatively, the enzymatic nucleic acid molecules can be expressed from DNA/RNA vectors that are delivered to specific cells. DNAzymes can be synthesized chemically or expressed endogenously *in vivo,* by means of a single stranded DNA vector or equivalent thereof.

In another embodiment, the nucleic acid molecule of the invention binds irreversibly to the HBV reverse transcriptase target, for example by covalent attachment of the nucleic molecule to the reverse transcriptase primer sequence. The covalent attachment can be accomplished by introducing chemical modifications into the nucleic acid molecule's (for example, decoy or aptamer) sequence that are capable of forming covalent bonds to the reverse transcriptase primer sequence.

In another embodiment, the nucleic acid molecule of the invention binds irreversibly to the HBV Enhancer I sequence target, for example, by covalent attachment of the nucleic acid molecule to the HBV Enhancer I sequence. The covalent attachment can be accomplished by introducing chemical modifications into the nucleic acid molecule's sequence that are capable of forming covalent bonds to the reverse transcriptase primer sequence.

In another embodiment, the type I interferon contemplated by the invention is interferon alpha, interferon beta, consensus interferon, polyethylene glycol interferon,

polyethylene glycol interferon alpha 2a, polyethylene glycol interferon alpha 2b, polyethylene glycol consensus interferon.

In one embodiment, the invention features a composition comprising type I interferon and a nucleic acid molecule of the inventionand a pharmaceutically acceptable carrier.

In another embodiment, the invention features a method of administering to a cell, for example a mammalian cell or human cell, a nucleic acid molecule of the invention independently or in conjunction with other therapeutic compounds, such as type I interferon or 3TC® (lamivudine), comprising contacting the cell with the nucleic acid molecule under conditions suitable for the administration.

In yet another embodiment, the invention features a method of administering to a cell, for example a mammalian cell or human cell, a nucleic acid molecule of the invention independently or in conjunction with other therapeutic compounds such as enzymatic nucleic acid molecules, antisense molecules, triplex forming oligonucleotides, 2,5-A chimeras, and/or RNAi, comprising contacting the cell with the nucleic acid molecule of the invention under conditions suitable for the administration.

In another embodiment, administration of a nucleic acid molecule of the invention is administered to a cell or patient in the presence of a delivery reagent, for example a lipid, cationic lipid, phospholipid, or liposome.

In one embodiment, the invention features novel nucleic acid-based techniques such as nucleic acid decoy molecules and/or aptamers, used alone or in combination with enzymatic nucleic acid molecules, antisense molecules, and/or RNAi, and methods for use to down regulate or modulate the expression of HBV RNA and/or replication of HBV.

In another embodiment, the invention features the use of one or more of the nucleic acid-based techniques to modulate the expression of the genes encoding HBV viral proteins. Specifically, the invention features the use of nucleic acid-based techniques to specifically modulate the expression of the HBV viral genome.

In another embodiment, the invention features the use of one or more of the nucleic acid-based techniques to modulate the activity, expression, or level of cellular proteins required for HBV replication. For example, the invention features the use of nucleic acid-based techniques to specifically modulate the activity of cellular proteins required for HBV replication.

In another embodiment, the invention features nucleic acid-based modulators(*e.g.*, nucleic acid decoy molecules, aptamers, enzymatic nucleic acid molecules (ribozymes),

antisense nucleic acids, triplex DNA, antisense nucleic acids containing RNA cleaving chemical groups) and methods for their use to down regulate or modulate reverse transcriptase activity and/or the expression of RNA (*e.g.*, HBV) capable of progression and/or maintenance of HBV infection, hepatocellular carcinoma, liver disease and failure.

In another embodiment, the invention features nucleic acid-based techniques (*e.g.*, nucleic acid decoy molecules, aptamers, enzymatic nuleic acid molecules (ribozymes), antisense nucleic acid molecules, triplex DNA, antisense nucleic acids containing RNA cleaving chemical groups) and methods for their use to down regulate or modulate reverse transcriptase activity and/or the expression of HBV RNA.

In another embodiment, the invention features nucleic acid-based modulators (*e.g.*, nucleic acid decoy molecules, aptamers, enzymatic nucleic acid molecules (ribozymes), antisense nucleic acids, triplex DNA, siRNA, dsRNA, antisense nucleic acids containing RNA cleaving chemical groups) and methods for their use to down regulate or modulate Enhancer I mediated transcription activity and/or the expression of DNA (*e.g.*, HBV) capable of progression and/or maintenance of HBV infection, hepatocellular carcinoma, liver disease and failure.

In another embodiment, the invention features nucleic acid-based techniques (*e.g.*, nucleic acid decoy molecules, aptamers, enzymatic nucleic acid molecules, antisense nucleic acid molecules, triplex DNA, siRNA, antisense nucleic acids containing DNA cleaving chemical groups) and methods for their use to down regulate or modulate Enhancer I mediated transcription activity and/or the expression of HBV DNA.

In another embodiment, the invention features a nucleic acid sensor molecule having an enzymatic nucleic acid domain and a sensor domain that interacts with an HBV peptide, protein, or polynucleotide sequence, for example, HBV reverse transcriptase, HBV reverse transcriptase primer, or the Enhancer I element of the HBV pregenomic RNA, wherein such interaction results in modulation of the activity of the enzymatic nucleic acid domain of the nucleic acid sensor molecule. In another embodiment, the invention features HBV-specific nucleic acid sensor molecules or allozymes, and methods for their use to down regulate or modulate the expression of HBV RNA capable of progression and/or maintenance of hepatitis, hepatocellular carcinoma, cirrhosis, and/or liver failure. In yet another embodiment, the enzymatic nucleic acid domain of a nucleic acid sensor molecule of the invention is a Hammerhead, Inozyme, G-cleaver, DNAzyme, Zinzyme, Amberzyme, or Hairpin enzymatic nucleic acid molecule.

In one embodiment, nucleic acid molecules of the invention are used to treat HBV-infected cells or a HBV-infected patient wherein the HBV is resistant or the patient does not

respond to treatment with 3TC® (Lamivudine), either alone or in combination with other therapies under conditions suitable for the treatment.

In another embodiment, nucleic acid molecules of the invention are used to treat HBV-infected cells or a HBV-infected patient, wherein the HBV is resistant or the patient does not respond to treatment with Interferon, for example Infergen®, either alone or in combination with other therapies under conditions suitable for the treatment.

The invention also relates to *in vitro* and *in vivo* systems, including, e.g., mammalian systems for screening inhibitors of HBV. In one embodiment, the invention features a mouse, for example a male or female mouse, implanted with HepG2.2.15 cells, wherein the mouse is susceptible to HBV infection and capable of sustaining HBV DNA expression. One embodiment of the invention provides a mouse implanted with HepG2.2.15 cells, wherein said mouse sustains the propagation of HEPG2.2.15 cells and HBV production.

In another embodiment, a mouse of the invention has been infected with HBV for at least one week to at least eight weeks, including, for example at least 4 weeks.

In yet another embodiment, a mouse of the invention, for example a male or female mouse, is an immunocompromised mouse, for example a nu/nu mouse or a scid/scid mouse.

In one embodiment, the invention features a method of producing a mouse of the invention, comprising injecting, for example by subcutaneous injection, HepG2.2.15 (Sells, *et al,*. 1987, *Proc Natl Acad Sci U S A.*, 84, 1005-1009) cells into the mouse under conditions suitable for the propagation of HepG2.2.15 cells in said mouse. HepG2.2.15 cells can be suspended in, for example, Delbecco's PBS solution including calcium and magnesium. In another embodiment, HepG2.2.15 cells are selected for antibiotic resistance and are then introduced into the mouse under conditions suitable for the propagation of HepG2.2.15 cells in said mouse. A non-limiting example of antibiotic resistant HepG2.2.15 cells include G418 antibiotic resistant HepG2.2.15 cells.

In another embodiment, the invention features a method of screening a compound for therapeutic activity against HBV, comprising administering the compound to a mouse of the invention and monitoring the the levels of HBV produced (e.g. by assaying for HBV DNA levels) in the mouse.

In one embodiment, a therapeutic compound or therapy contemplated by the invention is a lipid, steroid, peptide, protein, antibody, monoclonal antibody, humanized monoclonal antibody, small molecule, and/or isomers and analogs thereof, and/or a cell.

In one embodiment, a therapeutic compound or therapy contemplated by the invention is a nucleic acid molecule, for example a nucleic acid molecule, such as an enzymatic nucleic acid molecule, antisense nucleic acid molecule, allozyme, peptide nucleic acid, decoy, triplex oligonucleotide, dsRNA, ssRNA, RNAi, siRNA, aptamer, or 2,5-A chimera used alone or in combination with another therapy, for example antiviral therapy. Antiviral therapy can be, for example, treatment with 3TC® (Lamivudine) or interferon. Interferon can include, for example, consensus interferon or type I interferon. Type I interferon can include interferon alpha, interferon beta, consensus interferon, polyethylene glycol interferon, polyethylene glycol interferon alpha 2a, polyethylene glycol interferon alpha 2b, or polyethylene glycol consensus interferon.

In one embodiment, the invention features a non-human mammal implanted with HepG2.2.15 cells, wherein the non-human mammal is susceptible to HBV infection and capable of sustaining HBV DNA expression in the implanted HepG2.2.15 cells.

In another embodiment, a non-human mammal of the invention, for example a male or female non-human mammal, has been infected with HBV for at least one week to at least eight weeks, including for example at least four weeks.

In yet another embodiment, a non-human mammal of the invention is an immunocompromised mammal, for example a nu/nu mammal or a scid/scid mammal.

In one embodiment, the invention features a method of producing a non-human mammal comprising HepG2.2.15 cells comprising injecting, for example by subcutaneous injection, HepG2.2.15 cells into the non-human mammal under conditions suitable for the propagation of HepG2.2.15 cells in said non-human mammal.

In another embodiment, the invention features a method of screening a compound for therapeutic activity against HBV comprising administering the compound to a non-human mammal of the invention and monitoring the levels of HBV produced (e.g. by assaying for HBV DNA levels) in the non-human mammals.

In one embodiment, a therapeutic compound or therapy contemplated by the invention is a nucleic acid molecule, for example an enzymatic nucleic acid molecule, allozyme, antisense nucleic acid molecule, decoy, triplex oligonucleotide, dsRNA, ssRNA, RNAi, siRNA, or 2,5-A chimera used alone or in combination with another therapy, for example antiviral therapy.

Methods and chimeric immunocompromised heterologous non-human mammalian hosts, particularly mouse hosts, are provided for the expression of hepatitis B virus ("HBV").

In one embodiment, the chimeric hosts have transplanted viable, HepG2.2.15 cells in an immunocompromised host.

The non-human mammals contemplated by the invention are immunocompromised in normally inheriting the desired immune incapacity, or the desired immune incapacity can be created. For example, hosts with severe combined immunodeficiency, known as scid/scid hosts, are available. Rodentia, particularly mice, and equine, particularly horses, are presently available as scid/scid hosts, for example scid/scid mice and scid/scid rats. The scid/scid hosts lack functioning lymphocyte types, particularly B-cells and some T-cell types. In the scid/scid mouse hosts, the genetic defect appears to be a non-functioning recombinase, as the germline DNA is not rearranged to produce functioning surface immunoglobulin and T-cell receptors.

Any immunodeficient non-human mammals, e.g. mouse, can be used to generate the animal models described herein. The term "immunodeficient," as used herein, refers to a genetic alteration that impairs the animal's ability to mount an effective immune response. In this regard, an "effective immune response" is one which is capable of destroying invading pathogens such as (but not limited to) viruses, bacteria, parasites, malignant cells, and/or a xenogeneic or allogeneic transplant. In one embodiment, the immunodeficient mouse is a severe immunodeficient (SCID) mouse, which lacks recombinase activity that is necessary for the generation of immunoglobulin and functional T cell antigen receptors, and thus does not produce functional B and T lymphocytes. In another embodiment, the immunodeficient mouse is a nude mouse, which contains a genetic defect that results in the absence of a functional thymus, leading to T-cell and B-cell deficiencies. However, mice containing other immunodeficiencies (such as rag-1 or rag-2 knockouts, as described in Chen *et al.,* 1994, *Curr. Opin. Immunol.,* 6, 313-319 and Guidas *et al.,* 1995, *J. Exp. Med.,* 181, 1187-1195, or beige-nude mice, which also lack natural killer cells, as described in Kollmann *et al.,* 1993, *J. Exp. Med.*, 177, 821-832) can also be employed.

The introduction of HepG2.2.15 cells occurs with a host at an age less than about 25% of its normal lifespan, usually to 20% of the normal lifespan with mice, and the age will generally be of an age of about 3 to 10 weeks, more usually from about 4 to 8 weeks. The mice can be of either sex, can be neutered, and can be otherwise normal, except for the immunocompromised state, or they can have one or more mutations, which can be naturally occurring or as a result of mutagenesis.

In another embodiment, the mouse model described herein is used to evaluate the effectiveness of thetherapeutic compounds and methods. The terms "therapeutic compounds", "therapeutic methods" and "therapy" as used herein, encompass exogenous factors, such as dietary or environmental conditions, as well as pharmaceutical compositions

"drugs" and vaccines. In one embodiment, the therapeutic method is an immunotherapy, which can include the treatment of the HBV bearing animal with populations of HBV-reactive immune cells. The therapeutic method can also, or alternatively, be a gene therapy (i.e., a therapy that involves treatment of the HBV-bearing mouse with a cell population that has been manipulated to express one or more genes, the products of which can possess anti-viral activity), see for example The Development of Human Gene Therapy, Theodore Friedmann, Ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1999. Therapeutic compounds of the invention can comprise a drug or composition with pharmaceutical activity that can be used to treat illness or disease. A therapeutic method can comprise the use of a plurality of compounds in a mixture or a distinct entity. Examples of such compounds include nucleosides, nucleic acids, nucleic acid chimeras, RNA and DNA oligonucleotides, peptide nucleic acids, enzymatic nucleic acid molecules, antisense nucleic acid molecules, decoys, triplex oligonucleotides, ssDNA, dsRNA, ssRNA, siRNA, 2,5-A chimeras, lipids, steroids, peptides, proteins, antibodies, monoclonal antibodies (see for example Hall, 1995, Science, 270, 915-916), small molecules, and/or isomers and analogs thereof.

The methods of this invention can be used to treat human hepatitis B virus infections, which include productive virus infection, latent or persistent virus infection, and HBV-induced hepatocyte transformation. The utility can be extended to other species of HBV that infect non-human animals where such infections are of veterinary importance.

Preferred binding sites of the nucleic acid molecules of the invention include, but are not limited, to the primer binding site on HBV reverse transcriptase, the primer binding sequences of the HBV RNA, and/or the HBV Enhancer I region of HBV DNA.

This invention further relates to nucleic acid molecules that target RNA species of hepatitis C virus (HCV) and/or encoded by the HCV. In one embodiment, applicant describes enzymatic nucleic acid molecules that specifically cleave HCV RNA and the selection and function thereof. The invention further relates to compounds and chimeric molecules comprising nuclease activating activity. The invention also relates to compositions and methods for the cleavage of RNA using these nuclease activating compounds and chimeras. Nucleic acid molecules, nuclease activating compounds and chimeras, and compostions and methods of the invention can be used to treat diseases associated with HCV infection.

Due to the high sequence variability of the HCV genome, selection of nucleic acid molecules and nuclease activating compounds and chimeras for broad therapeutic applications preferably involve the conserved regions of the HCV genome. Thus, in one embodiment the present invention describes nucleic acid molecules that cleave the conserved

regions of the HCV genome. The invention further describes compounds and chimeric molecules that activate cellular nucleases that cleave HCV RNA, including concerved regions of the HCV genome. Examples of conserved regions of the HCV genome include but are not limited to the 5'-Non Coding Region (NCR), the 5'-end of the core protein coding region, and the 3'- NCR. HCV genomic RNA contains an internal ribosome entry site (IRES) in the 5'-NCR which mediates translation independently of a 5'-cap structure (Wang *et al.*, 1993, *J. Virol.*, 67, 3338-44). The full-length sequence of the HCV RNA genome is heterologous among clinically isolated subtypes, of which there are at least 15 (Simmonds, 1995, *Hepatology,* 21, 570-583), however, the 5'-NCR sequence of HCV is highly conserved across all known subtypes, most likely to preserve the shared IRES mechanism (Okamoto *et al.*, 1991, *J. General Virol.*, 72, 2697-2704). In general, enzymatic nucleic acid molecules and nuclease activating compounds, and chimeras that cleave sites located in the 5' end of the HCV genome are expected to block translation while nucleic acid molecules and nuclease activating compounds, and chimeras that cleave sites located in the 3' end of the genome are expected to block RNA replication. Therefore, one nucleic acid molecule, compound, or chimera can be designed to cleave all the different isolates of HCV. Enzymatic nucleic acid molecules and nuclease activating compounds, and chimeras designed against conserved regions of various HCV isolates enable efficient inhibition of HCV replication in diverse patient populations and ensure the effectiveness of the nucleic acid molecules and nuclease activating compounds, and chimeras against HCV quasi species which evolve due to mutations in the non-conserved regions of the HCV genome.

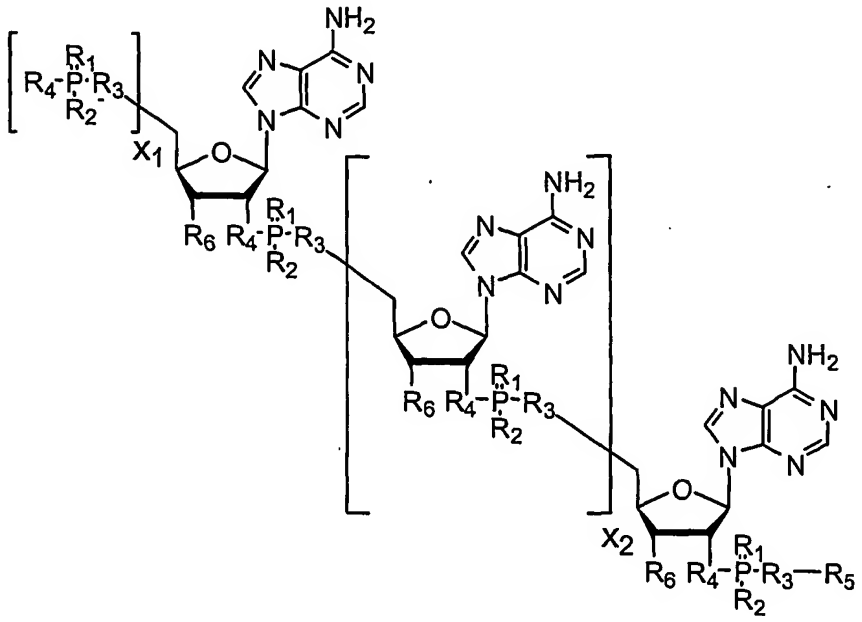
In one embodiment, the invention features an enzymatic nucleic acid molecule, preferably in the hammerhead, NCH (Inozyme), G-cleaver, amberzyme, zinzyme and/or DNAzyme motif, and the use thereof to down-regulate or inhibit the expression of HCV RNA.

In another embodiment, the invention features an enzymatic nucleic acid molecule, preferably in the hammerhead, Inozyme, G-cleaver, amberzyme, zinzyme and/or DNAzyme motif, and the use thereof to down-regulate or inhibit the expression of HCV minus strand RNA.

In yet another embodiment, the invention featues a nuclease activating compound and/or a chimera and the use thereof to down-regulate or inhibit the expression of HCV RNA.

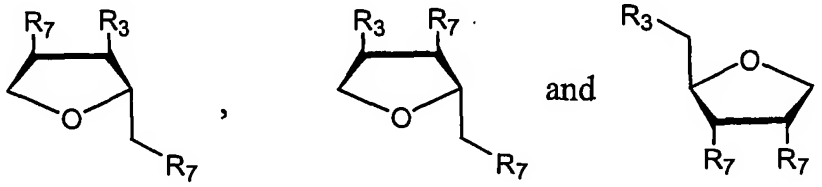
In another embodiment, the invention featues the use of a nuclease activating compound and/or a chimera to inhibit the expression of HCVminus strand RNA.

In one embodiment, the invention features a compound having formula I:



wherein X_1 is an integer selected from the group consisting of 1, 2, and 3; X_2 is an integer greater than or equal to 1; R_6 is independantly selected from the group including H, OH, NH_2, O NH_2, alkyl, S-alkyl, O-alkyl, O-alkyl-S-alkyl, O-alkoxyalkyl, allyl, O-allyl, and fluoro; each R_1 and R_2 are independantly selected from the group consisting of O and S; each R_3 and R_4 are independantly selected from the group consisting of O, N, and S; and R_5 is selected from the group consisting of alkyl, alkylamine, an oligonucleotide having any of SEQ ID NOS. 11343-16182, an oligonucleotide having a sequence complementary to a sequence selected from the group including SEQ ID NOS. 2594-7433, and abasic moiety.

In another embodiment, the abasic moiety of the instant invention is selected from the group consisting of:



wherein R_3 is selected from the group consisting of O, N, and S, and R_7 is independently selected from the group consisting of H, OH, NH2, O-NH2, alkyl, S-alkyl, O-alkyl, O-alkyl-S-alkyl, O-alkoxyalkyl, allyl, O-allyl, fluoro, oligonucleotide, alkyl, alkylamine and abasic moiety.

In another embodiment, the oligonucleotide R_5 of Formula I having a sequence complementary to a sequence selected from the group consisting of SEQ ID NOS. 2594-7433 is an enzymatic nucleic acid molecule.

In yet another embodiment, the oligonucleotide R_5 of Formula I having a sequence complementary to a sequence selected from the group consisting of SEQ ID NOS. 2594-7433 is an antisense nucleic acid molecule.

In another embodiment, the oligonucleotide R_5 of Formula I having a sequence complementary to a sequence selected from the group consisting of SEQ ID NOS. 2594-7433 is an enzymatic nucleic acid molecule selected from the group consisting of Hammerhead, Inozyme, G-cleaver, DNAzyme, Amberzyme, and Zinzyme motifs.

In another embodiment, the Inozyme enzymatic nucleic acid molecule of the instant invention comprises a stem II region of length greater than or equal to 2 base pairs.

In one embodiment, the oligonucleotide R_5 of Formula I having a sequence complementary to a sequence selected from the group consisting of SEQ ID NOS. 2594-7433 is an enzymatic nucleic acid comprising between 12 and 100 bases complementary to an RNA derived from HCV.

In another embodiment, the oligonucleotide R_5 of Formula I having a sequence complementary to a sequence selected from the group consisting of SEQ ID NOS. 2594-7433 is an enzymatic nucleic acid comprising between 14 and 24 bases complementary to said RNA derived from HCV.

In one embodiment, the oligonucleotide R_5 of Formula I having a sequence complementary to a sequence selected from the group consisting of SEQ ID NOS. 2594-7433 is an antisense nucleic acid comprising between 12 and 100 bases complementary to an RNA derived from HCV.

In another embodiment, the oligonucleotide R_5 of Formula I having a sequence complementary to a sequence selected from the group consisting of SEQ ID NOS. 2594-7433 is an antisense nucleic acid comprising between 14 and 24 bases complementary to said RNA derived from HCV.

In another embodiment, the invention features a composition comprising a compound of Formula I, in a pharmaceutically acceptable carrier.

In yet another embodiment, the invention features a mammalian cell comprising a compound of Formula I. For example, the mammalian cell comprising a compound of Formula I can be a human cell.

In one embodiment, the invention features a method for the treatment of cirrhosis, liver failure, hepatocellular carcinoma, or a condition associated with HCV infection comprising

the step of administering to a patient a compound of Formula I under conditions suitable for said treatment.

In another embodiment, the invention features a method of treatment of a patient having a condition associated with HCV infection comprising contacting cells of said patient with a compound having Formula I, and further comprising the use of one or more drug therapies under conditions suitable for said treatment. For example, the other therapies of the instant invention can be selected from the group consisting of type I interferon, interferon alpha, interferon beta, consensus interferon, polyethylene glycol interferon, polyethylene glycol interferon alpha 2a, polyethylene glycol interferon alpha 2b, polyethylene glycol consensus interferon, treatment with an enzymatic nucleic acid molecule, and treatment with an antisense molecule.

In another embodiment, the other therapies of the instant invention, for example type I interferon, interferon alpha, interferon beta, consensus interferon, polyethylene glycol interferon, polyethylene glycol interferon alpha 2a, polyethylene glycol interferon alpha 2b, polyethylene glycol consensus interferon, treatment with an enzymatic nucleic acid molecule, and treatment with an antisense nucleic acid molecule, and the compound having Formula I are administered separately in separate pharmaceutically acceptable carriers.

In yet another embodiment, the other therapies of the instant invention, for example type I interferon, interferon alpha, interferon beta, consensus interferon, polyethylene glycol interferon, polyethylene glycol interferon alpha 2a, polyethylene glycol interferon alpha 2b, polyethylene glycol consensus interferon, treatment with an enzymatic nucleic acid molecule, and treatment with an antisense nucleic acid molecule, and the compound having Formula I are administered simultaneously in a pharmaceutically acceptable carrier. The invention features a composition comprising a compound of Formula I and one or more of the above-listed compounds in a pharmaceutically acceptable carrier.

In yet another embodiment, the invention features a method for inhibiting HCV replication in a mammalian cell comprising the step of administering to said cell a compound having Formula I under conditions suitable for said inhibition.

In another embodiment, the invention features a method of cleaving a separate RNA molecule (i.e., HCV RNA or RNA necessary for HCV replication) comprising contacting a compound having Formula I with the separate RNA molecule under conditions suitable for the cleavage of the separate RNA molecule. In one example, the method of cleaving a separate RNA molecule is carried out in the presence of a divalent cation, for example Mg2+.

In yet another embodiment, the method of cleaving a separate RNA molecule of the invention is carried out in the presence of a protein nuclease, for example RNAse L.

In one embodiment, a compound having Formula I is chemically synthesized. In one embodiment, a compound having Formula I comprises at least one 2'-sugar modification, at least one nucleic acid base modification, and/or at least one phosphate modification.

The nucleic acid-based modulators of the invention are added directly, or can be complexed with cationic lipids, packaged within liposomes, or otherwise delivered to target cells or tissues. The nucleic acid or nucleic acid complexes can be locally administered to relevant tissues *ex vivo*, or *in vivo* through injection, infusion pump or stent, with or without their incorporation in biopolymers. In particular embodiments, the nucleic acid molecules of the invention comprise sequences shown in **Tables IV-XI, XIV-XV and XVIII-XXIII**. Examples of such nucleic acid molecules consist essentially of sequences defined in the tables.

The nucleic acid-based inhibitors, nuclease activating compounds and chimeras of the invention are added directly, or can be complexed with cationic lipids, packaged within liposomes, or otherwise delivered to target cells or tissues. The nucleic acid or nucleic acid complexes, and nuclease activating compounds or chimeras can be locally administered to relevant tissues ex vivo, or in vivo through injection or infusion pump, with or without their incorporation in biopolymers. In preferred embodiments, the enzymatic nucleic acid inhibitors, and nuclease activating compounds or chimeras comprise sequences, which are complementary to the substrate sequences in **Tables XVIII, XIX, XX and XXIII**. Examples of such enzymatic nucleic acid molecules also are shown in **Tables XVIII, XIX, XX, XXI and XXIII**. Examples of such enzymatic nucleic acid molecules consist essentially of sequences defined in these tables. In additional embodiments, the enzymatic nucleic acid inhibitors of the invention that comprise sequences which are complementary to the substrate sequences in **Tables XVIII, XIX, XX and XXIII** are covalently attached to nuclease activating compound or chimeras of the invention, for example a compound having Formula I.

In yet another embodiment, the invention features antisense nucleic acid molecules and 2-5A chimera including sequences complementary to the substrate sequences shown in **Tables XVIII, XIX, XX and XXIII**. Such nucleic acid molecules can include sequences as shown for the binding arms of the enzymatic nucleic acid molecules in **Tables XVIII, XIX, XX, XXI and XXIII**. Similarly, triplex molecules can be provided targeted to the corresponding DNA target regions, and containing the DNA equivalent of a target sequence or a sequence complementary to the specified target (substrate) sequence. Typically, antisense molecules are complementary to a target sequence along a single contiguous

sequence of the antisense molecule. However, in certain embodiments, an antisense molecule can bind to substrate such that the substrate molecule forms a loop, and/or an antisense molecule can bind such that the antisense molecule forms a loop. Thus, the antisense molecule can be complementary to two (or even more) non-contiguous substrate sequences or two (or even more) non-contiguous sequence portions of an antisense molecule can be complementary to a target sequence or both.

In one embodiment, the invention features nucleic acid molecules and nuclease activating compounds or chimeras that inhibit gene expression and/or viral replication. These chemically or enzymatically synthesized nucleic acid molecules can contain substrate binding domains that bind to accessible regions of their target mRNAs. The nucleic acid molecules also contain domains that catalyze the cleavage of RNA. The enzymatic nucleic acid molecules are preferably molecules of the hammerhead, Inozyme, DNAzyme, Zinzyme, Amberzyme, and/or G-cleaver motifs. Upon binding, the enzymatic nucleic acid molecules cleave the target mRNAs, preventing translation and protein accumulation. In the absence of the expression of the target gene, HCV gene expression and/or replication is inhibited.

In another aspect, the invention provides mammalian cells containing one or more nucleic acid molecules and/or expression vectors of this invention. The one or more nucleic acid molecules can independently be targeted to the same or different sites.

In one embodiment, nucleic acid decoys, aptamers, siRNA, enzymatic nucleic acids or antisense molecules that interact with target protein and/or RNA molecules and modulate HBV (specifically HBV reverse transcriptase, or transcription of HBV genomic DNA) activity are expressed from transcription units inserted into DNA or RNA vectors. The recombinant vectors are preferably DNA plasmids or viral vectors. Decoys, aptamers, enzymatic nucleic acid or antisense expressing viral vectors can be constructed based on, but not limited to, adeno-associated virus, retrovirus, adenovirus, or alphavirus. Preferably, the recombinant vectors capable of expressing the decoys, aptamers, enzymatic nucleic acids or antisense are delivered as described above, and persist in target cells. Alternatively, viral vectors can be used that provide for transient expression of decoys, aptamers, siRNA, enzymatic nucleic acids or antisense. Such vectors can be repeatedly administered as necessary. Once expressed, the decoys, aptamers, enzymatic nucleic acids or antisense bind to the target protein and/or RNA and modulate its function or expression. Delivery of decoy, aptamer, siRNA, enzymatic nucleic acid or antisense expressing vectors can be systemic, such as by intravenous or intramuscular administration, by administration to target cells ex-planted from the patient followed by reintroduction into the patient, or by any other means that would allow for introduction into the desired target cell. DNA based nucleic acid

molecules of the invention can be expressed via the use of a single stranded DNA intracellular expression vector.

In one embodiment, nucleic acid molecules and nuclease activating compounds or chimeras are added directly, or can be complexed with cationic lipids, packaged within liposomes, or otherwise delivered to target cells. The nucleic acid or nucleic acid complexes can be locally administered to relevant tissues ex vivo, or in vivo through injection, infusion pump or stent, with or without their incorporation in biopolymers. In another preferred embodiment, the nucleic acid molecule, nuclease activating compound or chimera is administered to the site of HBV or HCV activity (e.g., hepatocytes) in an appropriate liposomal vehicle.

In another embodiment, nucleic acid molecules that cleave target molecules and inhibit HCV activity are expressed from transcription units inserted into DNA or RNA vectors. The recombinant vectors are preferably DNA plasmids or viral vectors. Nucleic acid molecule expressing viral vectors can be constructed based on, but not limited to, adeno-associated virus, retrovirus, adenovirus, or alphavirus. Preferably, the recombinant vectors capable of expressing the nucleic acid molecules are delivered as described above, and persist in target cells. Alternatively, viral vectors can be used that provide for transient expression of nucleic acid molecules. Such vectors can be repeatedly administered as necessary. Once expressed, the nucleic acid molecules cleave the target mRNA. Delivery of enzymatic nucleic acid molecule expressing vectors can be systemic, such as by intravenous or intramuscular administration, by administration to target cells ex-planted from the patient followed by reintroduction into the patient, or by any other means that would allow for introduction into the desired target cell (for a review see Couture and Stinchcomb, 1996, *TIG.*, 12, 510). In another aspect of the invention, nucleic acid molecules that cleave target molecules and inhibit viral replication are expressed from transcription units inserted into DNA, RNA, or viral vectors. Preferably, the recombinant vectors capable of expressing the nucleic acid molecules are locally delivered as described above, and transiently persist in smooth muscle cells. However, other mammalian cell vectors that direct the expression of RNA can be used for this purpose.

The nucleic acid molecules of the instant invention, individually, or in combination or in conjunction with other drugs, and/or therapies can be used to treat diseases or conditions discussed herein. For example, to treat a disease or condition associated with the levels of HBV or HCV, the nucleic acid molecules can be administered to a patient or can be administered to other appropriate cells evident to those skilled in the art, individually or in combination with one or more drugs under conditions suitable for the treatment.

In a further embodiment, the described molecules, such as decoys, aptamers, antisense, enzymatic nucleic acids, or nuclease activating compounds and chimeras can be used in combination with other known treatments to treat conditions or diseases discussed above. For example, the described molecules could be used in combination with one or more known therapeutic agents to treat HBV infection, HCV infection, hepatitis, hepatocellular carcinoma, cancer, cirrhosis, and liver failure. Such therapeutic agents can include, but are not limited to, nucleoside analogs selected from the group comprising Lamivudine (3TC®), L-FMAU, and/or adefovir dipivoxil (for a review of applicable nucleoside analogs, see Colacino and Staschke, 1998, *Progress in Drug Research*, 50, 259-322). Immunomodulators selected from the group comprising Type 1 Interferon, therapeutic vaccines, steriods, and 2'-5' oligoadenylates (for a review of 2'-5' Oligoadenylates, see Charubala and Pfleiderer, 1994, *Progress in Molecular and Subcellular Biology*, 14, 113-138).

Nucleic acid molecules, nuclease activating compounds and chimeras of the invention, individually, or in combination or in conjunction with other drugs, can be used to treat diseases or conditions discussed above. For example, to treat a disease or condition associated with HBV or HCV levels, the patient can be treated, or other appropriate cells can be treated, as is evident to those skilled in the art.

In a further embodiment, the described molecules can be used in combination with other known treatments to treat conditions or diseases discussed above. For example, the described molecules can be used in combination with one or more known therapeutic agents to treat liver failure, hepatocellular carcinoma, cirrhosis, and/or other disease states associated with HBV or HCV infection. Additional known therapeutic agents are those comprising antivirals, interferons, and/or antisense compounds.

The term "inhibit" or "down-regulate" as used herein refers to the expression of the gene, or level of RNAs or equivalent RNAs encoding one or more protein subunits or components, or activity of one or more protein subunits or components, such as HBV protein or proteins, is reduced below that observed in the absence of the therapies of the invention. In one embodiment, inhibition or down-regulation with enzymatic nucleic acid molecule preferably is below that level observed in the presence of an enzymatically inactive or attenuated molecule that is able to bind to the same site on the target RNA, but is unable to cleave that RNA. In another embodiment, inhibition or down-regulation with antisense oligonucleotides is preferably below that level observed in the presence of, for example, an oligonucleotide with scrambled sequence or with mismatches. In another embodiment, inhibition or down-regulation of HBV with the nucleic acid molecule of the instant invention is greater in the presence of the nucleic acid molecule than in its absence.

The term "up-regulate" as used herein refers to the expression of the gene, or level of RNAs or equivalent RNAs encoding one or more protein subunits or components, or activity of one or more protein subunits or components, such as HBV or HCV protein or proteins, is greater than that observed in the absence of the therapies of the invention. For example, the expression of a gene, such as HBV or HCV genes, can be increased in order to treat, prevent, ameliorate, or modulate a pathological condition caused or exacerbated by an absence or low level of gene expression.

The term "modulate" as used herein refers to the expression of the gene, or level of RNAs or equivalent RNAs encoding one or more protein subunits or components, or activity of one or more proteins is up-regulated or down-regulated, such that the expression, level, or activity is greater than or less than that observed in the absence of the therapies of the invention.

The term "decoy " as used herein refers to a nucleic acid molecule, for example RNA or DNA, or aptamer that is designed to preferentially bind to a predetermined ligand. Such binding can result in the inhibition or activation of a target molecule. A decoy or aptamer can compete with a naturally occurring binding target for the binding of a specific ligand. For example, it has been shown that over-expression of HIV trans-activation response (TAR) RNA can act as a "decoy" and efficiently binds HIV tat protein, thereby preventing it from binding to TAR sequences encoded in the HIV RNA (Sullenger *et al.*, 1990, Cell, 63, 601-608). This is but a specific example and those in the art will recognize that other embodiments can be readily generated using techniques generally known in the art, see for example Gold *et al.*, 1995, *Annu. Rev. Biochem.*, 64, 763; Brody and Gold, 2000, *J. Biotechnol.*, 74, 5; Sun, 2000, *Curr. Opin. Mol. Ther.*, 2, 100; Kusser, 2000, *J. Biotechnol.*, 74, 27; Hermann and Patel, 2000, *Science*, 287, 820; and Jayasena, 1999, *Clinical Chemistry*, 45, 1628. Similarly, a decoy can be designed to bind to HBV or HCV proteins and block the binding of HBV or HCV DNA or RNA or a decoy can be designed to bind to HBV or HCV proteins and prevent molecular interaction with the HBV or HCV proteins.

By "aptamer" or "nucleic acid aptamer" as used herein is meant a nucleic acid molecule that binds specifically to a target molecule wherein the nucleic acid molecule has sequence that is distinct from sequence recognized by the target molecule in its natural setting. Alternately, an aptamer can be a nucleic acid molecule that binds to a target molecule where the target molecule does not naturally bind to a nucleic acid. The target molecule can be any molecule of interest. For example, the aptamer can be used to bind to a ligand-binding domain of a protein, thereby preventing interaction of the naturally occurring ligand with the protein. This is a non-limiting example and those in the art will recognize that other embodiments can be readily generated using techniques generally known in the art, see for

example Gold *et al.*, 1995, *Annu. Rev. Biochem.*, 64, 763; Brody and Gold, 2000, *J. Biotechnol.*, 74, 5; Sun, 2000, *Curr. Opin. Mol. Ther.*, 2, 100; Kusser, 2000, *J. Biotechnol.*, 74, 27; Hermann and Patel, 2000, *Science*, 287, 820; and Jayasena, 1999, *Clinical Chemistry*, 45, 1628.

By "enzymatic nucleic acid molecule" is meant a nucleic acid molecule that has complementarity in a substrate binding region to a specified gene target, and also has an enzymatic activity which is active to specifically cleave a target RNA molecule. That is, the enzymatic nucleic acid molecule is able to intermolecularly cleave a RNA molecule and thereby inactivate a target RNA molecule. These complementary regions allow sufficient hybridization of the enzymatic nucleic acid molecule to a target RNA molecule and thus permit cleavage. One hundred percent complementarity is preferred, but complementarity as low as 50-75% may also be useful in this invention (see for example Werner and Uhlenbeck, 1995, *Nucleic Acids Research*, 23, 2092-2096; Hammann *et al.*, 1999, *Antisense and Nucleic Acid Drug Dev.*, 9, 25-31). The nucleic acids can be modified at the base, sugar, and/or phosphate groups. The term enzymatic nucleic acid is used interchangeably with phrases such as ribozymes, catalytic RNA, enzymatic RNA, catalytic DNA, aptazyme or aptamer-binding ribozyme, regulatable ribozyme, catalytic oligonucleotides, nucleozyme, DNAzyme, RNA enzyme, endoribonuclease, endonuclease, minizyme, leadzyme, oligozyme or DNA enzyme. All of these terminologies describe nucleic acid molecules with enzymatic activity. The specific enzymatic nucleic acid molecules described in the instant application are not limiting in the invention and those skilled in the art will recognize that all that is important in an enzymatic nucleic acid molecule of this invention is that it have a specific substrate binding site which is complementary to one or more of the target nucleic acid regions, and that it have nucleotide sequences within or surrounding that substrate binding site which impart a nucleic acid cleaving activity to the molecule (Cech et al., U.S. Patent No. 4,987,071; Cech et al., 1988, JAMA 260:20 3030-4).

By "nucleic acid molecule" as used herein is meant a molecule comprising nucleotides. The nucleic acid can be single, double, or multiple stranded and can comprise modified or unmodified nucleotides or non-nucleotides or various mixtures and combinations thereof.

By "enzymatic portion" or "catalytic domain" is meant that portion/region of the enzymatic nucleic acid molecule essential for cleavage of a nucleic acid substrate (for example see **Figures 1-5**).

By "substrate binding arm" or "substrate binding domain" is meant that portion/region of a ribozyme which is complementary to (*i.e.*, able to base-pair with) a portion of its substrate. Generally, such complementarity is 100%, but can be less if desired. For example, as few as 10 bases out of 14 may be base-paired (see for example Werner and Uhlenbeck,

1995, *Nucleic Acids Research,* 23, 2092-2096; Hammann *et al.,* 1999, *Antisense and Nucleic Acid Drug Dev.,* 9, 25-31). Such arms are shown generally in **Figures 1-5**. That is, these arms contain sequences within a ribozyme which are intended to bring ribozyme and target RNA together through complementary base-pairing interactions. The ribozyme of the invention can have binding arms that are contiguous or non-contiguous and may be of varying lengths. The length of the binding arm(s) are preferably greater than or equal to four nucleotides and of sufficient length to stably interact with the target RNA; specifically 12-100 nucleotides; more specifically 14-24 nucleotides long (see for example Werner and Uhlenbeck, *supra;* Hamman *et al., supra;* Hampel *et al.,* EP0360257; Berzal-Herrance *et al.,* 1993, *EMBO J.,* 12, 2567-73). If two binding arms are chosen, the design is such that the length of the binding arms are symmetrical (*i.e.,* each of the binding arms is of the same length; *e.g.,* five and five nucleotides, six and six nucleotides or seven and seven nucleotides long) or asymmetrical (*i.e.,* the binding arms are of different length; *e.g.,* six and three nucleotides; three and six nucleotides long; four and five nucleotides long; four and six nucleotides long; four and seven nucleotides long; and the like).

By "nuclease activating compound" is meant a compound, for example a compound having Formula I, that activates the cleavage of an RNA by a nuclease. The nuclease can comprise RNAse L. By "nuclease activating chimera" or "chimera" is meant a nuclease activating compound, for example a compound having Formula I, that is attached to a nulceic acid molecule, for example a nucleic acid molecule that binds preferentially to a target RNA. These chimeric nucleic acid molecules can comprise a nuclease activating compound and an antisense nucleic acid molecule, for example a 2',5'-oligoadenylate antisense chimera, or an enzymatic nucleic acid moleucle, for example a 2',5'-oligoadenylate enzymatic nucleic acid chimera.

By "Inozyme" or "NCH" motif or configuration is meant, an enzymatic nucleic acid molecule comprising a motif as is generally described as NCH Rz in Ludwig *et al.,* International PCT Publication No. WO 98/58058 and US Patent Application Serial No. 08/878,640. Inozymes possess endonuclease activity to cleave RNA substrates having a cleavage triplet NCH/, where N is a nucleotide, C is cytidine and H is adenosine, uridine or cytidine, and / represents the cleavage site. Inozymes can also possess endonuclease activity to cleave RNA substrates having a cleavage triplet NCN/, where N is a nucleotide, C is cytidine, and / represents the cleavage site.

By "G-cleaver" motif or configuration is meant, an enzymatic nucleic acid molecule comprising a motif as is generally described in Eckstein *et al.,* US 6,127,173 and in Kore *et al.,* 1998, *Nucleic Acids Research* 26, 4116-4120. G-cleavers possess endonuclease activity

to cleave RNA substrates having a cleavage triplet NYN/, where N is a nucleotide, Y is uridine or cytidine and / represents the cleavage site. G-cleavers can be chemically modified.

By "zinzyme" motif or configuration is meant, an enzymatic nucleic acid molecule comprising a motif as is generally described in Beigelman *et al.,* International PCT publication No. WO 99/55857 and US Patent Application Serial No. 09/918,728. Zinzymes possess endonuclease activity to cleave RNA substrates having a cleavage triplet including but not limited to, YG/Y, where Y is uridine or cytidine, and G is guanosine and / represents the cleavage site. Zinzymes can be chemically modified to increase nuclease stability through various substitutions, including substituting 2'-O-methyl guanosine nucleotides for guanosine nucleotides. In addition, differing nucleotide and/or non-nucleotide linkers can be used to substitute the 5'-gaaa-2' loop of the motif. Zinzymes represent a non-limiting example of an enzymatic nucleic acid molecule that does not require a ribonucleotide (2'-OH) group within its own nucleic acid sequence for activity.

By "amberzyme" motif or configuration is meant, an enzymatic nucleic acid molecule comprising a motif as is generally described in Beigelman *et al.,* International PCT publication No. WO 99/55857 and US Patent Application Serial No. 09/476,387. Amberzymes possess endonuclease activity to cleave RNA substrates having a cleavage triplet NG/N, where N is a nucleotide, G is guanosine, and / represents the cleavage site. Amberzymes can be chemically modified to increase nuclease stability. In addition, differing nucleoside and/or non-nucleoside linkers can be used to substitute the 5'-gaaa-3' loops of the motif. Amberzymes represent a non-limiting example of an enzymatic nucleic acid molecule that does not require a ribonucleotide (2'-OH) group within its own nucleic acid sequence for activity.

By 'DNAzyme' is meant, an enzymatic nucleic acid molecule that does not require the presence of a 2'-OH group within its own nucleic acid sequence for activity. In particular embodiments, the enzymatic nucleic acid molecule can have an attached linker or linkers or other attached or associated groups, moieties, or chains containing one or more nucleotides with 2'-OH groups. DNAzymes can be synthesized chemically or expressed endogenously *in vivo,* by means of a single stranded DNA vector or equivalent thereof. Non-limiting examples of DNAzymes are generally reviewed in Usman *et al.,* US patent No., 6,159,714; Chartrand *et al.,* 1995, *NAR* 23, 4092; Breaker *et al.,* 1995, *Chem. Bio.* 2, 655; Santoro *et al.,* 1997, *PNAS* 94, 4262; Breaker, 1999, *Nature Biotechnology,* 17, 422-423; and Santoro *et. al.,* 2000, *J. Am. Chem. Soc.,* 122, 2433-39. The "10-23" DNAzyme motif is one particular type of DNAzyme that was evolved using *in vitro* selection as generally described in Joyce *et al.,* US 5,807,718 and Santoro *et al., supra.* Additional DNAzyme motifs can be selected for

using techniques similar to those described in these references, and hence, are within the scope of the present invention.

By "nucleic acid sensor molecule" or "allozyme" as used herein is meant a nucleic acid molecule comprising an enzymatic domain and a sensor domain, where the enzymatic nucleic acid domain's ability to catalyze a chemical reaction is dependent on the interaction with a target signaling molecule, such as a nucleic acid, polynucleotide, oligonucleotide, peptide, polypeptide, or protein, for example HBV RT, HBV RT primer, or HBV Enhancer I sequence. The introduction of chemical modifications, additional functional groups, and/or linkers, to the nucleic acid sensor molecule can provide enhanced catalytic activity of the nucleic acid sensor molecule, increased binding affinity of the sensor domain to a target nucleic acid, and/or improved nuclease/chemical stability of the nucleic acid sensor molecule, and are hence within the scope of the present invention (see for example Usman *et al.,* US Patent Application No. 09/877,526, George *et al.,* US Patent Nos. 5,834,186 and 5,741,679, Shih *et al.,* US Patent No. 5,589,332, Nathan *et al.,* US Patent No 5,871,914, Nathan and Ellington, International PCT publication No. WO 00/24931, Breaker *et al.,* International PCT Publication Nos. WO 00/26226 and 98/27104, and Sullenger *et al.,* US Patent Application Serial No. 09/205,520).

By "sensor component" or "sensor domain" of the nucleic acid sensor molecule as used herein is meant, a nucleic acid sequence (e.g., RNA or DNA or analogs thereof) which interacts with a target signaling molecule, for example a nucleic acid sequence in one or more regions of a target nucleic acid molecule or more than one target nucleic acid molecule, and which interaction causes the enzymatic nucleic acid component of the nucleic acid sensor molecule to either catalyze a reaction or stop catalyzing a reaction. In the presence of target signaling molecule of the invention, such as HBV RT, HBV RT primer, or HBV Enhancer I sequence, the ability of the sensor component, for example, to modulate the catalytic activity of the nucleic acid sensor molecule, is altered or diminished in a manner that can be detected or measured. The sensor component can comprise recognition properties relating to chemical or physical signals capable of modulating the nucleic acid sensor molecule via chemical or physical changes to the structure of the nucleic acid sensor molecule. The sensor component can be derived from a naturally occurring nucleic acid binding sequence, for example, RNAs that bind to other nucleic acid sequences *in vivo.* Alternately, the sensor component can be derived from a nucleic acid molecule (aptamer), which is evolved to bind to a nucleic acid sequence within a target nucleic acid molecule. The sensor component can be covalently linked to the nucleic acid sensor molecule, or can be non-covalently associated. A person skilled in the art will recognize that all that is required is that the sensor component is able to selectively modulate the activity of the nucleic acid sensor molecule to catalyze a reaction.

By "target molecule" or "target signaling molecule" is meant a molecule capable of interacting with a nucleic acid sensor molecule, specifically a sensor domain of a nucleic acid sensor molecule, in a manner that causes the nucleic acid sensor molecule to be active or inactive. The interaction of the signaling agent with a nucleic acid sensor molecule can result in modification of the enzymatic nucleic acid component of the nucleic acid sensor molecule via chemical, physical, topological, or conformational changes to the structure of the molecule, such that the activity of the enzymatic nucleic acid component of the nucleic acid sensor molecule is modulated, for example is activated or inactivated. Signaling agents can comprise target signaling molecules such as macromolecules, ligands, small molecules, metals and ions, nucleic acid molecules including but not limited to RNA and DNA or analogs thereof, proteins, peptides, antibodies, polysaccharides, lipids, sugars, microbial or cellular metabolites, pharmaceuticals, and organic and inorganic molecules in a purified or unpurified form, for example HBV RT or HBV RT primer.

By "sufficient length" is meant a nucleic acid molecule long enough to provide the intended function under the expected condition. For example, a nucleic acid molecule of the invention needs to be of "sufficient length" to provide stable binding to a target site under the expected binding conditions and environment. In another non-limiting example, for the binding arms of an enzymatic nucleic acid, "sufficient length" means that the binding arm sequence is long enough to provide stable binding to a target site under the expected reaction conditions and environment. The binding arms are not so long as to prevent useful turnover of the nucleic acid molecule. By "stably interact" is meant interaction of the oligonucleotides with target nucleic acid (*e.g.*, by forming hydrogen bonds with complementary nucleotides in the target under physiological conditions) that is sufficient for the intended purpose (e.g., cleavage of target RNA by an enzyme).

By "equivalent" RNA to HBV or HCV is meant to include those naturally occurring RNA molecules having homology (partial or complete) to HBV or HCV proteins or encoding for proteins with similar function as HBV or HCV in various organisms, including human, rodent, primate, rabbit, pig, protozoans, fungi, plants, and other microorganisms and parasites. The equivalent RNA sequence also includes in addition to the coding region, regions such as 5'-untranslated region, 3'-untranslated region, introns, intron-exon junction and the like.

The term "component" of HBV or HCV as used herein refers to a peptide or protein subunit expressed from a HBV or HCV gene.

By "homology" is meant the nucleotide sequence of two or more nucleic acid molecules is partially or completely identical.

By "antisense nucleic acid", it is meant a non-enzymatic nucleic acid molecule that binds to target RNA by means of RNA-RNA or RNA-DNA or RNA-PNA (protein nucleic acid; Egholm *et al.,* 1993 *Nature* 365, 566) interactions and alters the activity of the target RNA (for a review, see Stein and Cheng, 1993 *Science* 261, 1004 and Woolf *et al.,* US patent No. 5,849,902). Typically, antisense molecules are complementary to a target sequence along a single contiguous sequence of the antisense molecule. However, in certain embodiments, an antisense molecule can bind to substrate such that the substrate molecule forms a loop, and/or an antisense molecule can bind such that the antisense molecule forms a loop. Thus, the antisense molecule can be complementary to two or more non-contiguous substrate sequences or two or more non-contiguous sequence portions of an antisense molecule can be complementary to a target sequence, or both. For a review of current antisense strategies, see Schmajuk *et al.,* 1999, *J. Biol. Chem.,* 274, 21783-21789, Delihas *et al.,* 1997, *Nature,* 15, 751-753, Stein *et al.,* 1997, *Antisense N. A. Drug Dev.,* 7, 151, Crooke, 2000, *Methods Enzymol.,* 313, 3-45; Crooke, 1998, *Biotech. Genet. Eng. Rev.,* 15, 121-157, Crooke, 1997, *Ad. Pharmacol.,* 40, 1-49. Antisense molecules of the instant invention can include 2-5A antisense chimera molecules. In addition, antisense DNA can be used to target RNA by means of DNA-RNA interactions, thereby activating RNase H, which digests the target RNA in the duplex. The antisense oligonucleotides can comprise one or more RNAse H activating region that is capable of activating RNAse H cleavage of a target RNA. Antisense DNA can be synthesized chemically or expressed via the use of a single stranded DNA expression vector or equivalent thereof.

By "RNase H activating region" is meant a region (generally greater than or equal to 4-25 nucleotides in length, preferably from 5-11 nucleotides in length) of a nucleic acid molecule capable of binding to a target RNA to form a non-covalent complex that is recognized by cellular RNase H enzyme (see for example Arrow *et al.,* US 5,849,902; Arrow *et al.,* US 5,989,912). The RNase H enzyme binds to the nucleic acid molecule-target RNA complex and cleaves the target RNA sequence. The RNase H activating region comprises, for example, phosphodiester, phosphorothioate (for example, at least four of the nucleotides are phosphorothiote substitutions; more specifically, 4-11 of the nucleotides are phosphorothiote substitutions), phosphorodithioate, 5'-thiophosphate, or methylphosphonate backbone chemistry or a combination thereof. In addition to one or more backbone chemistries described above, the RNase H activating region can also comprise a variety of sugar chemistries. For example, the RNase H activating region can comprise deoxyribose, arabino, fluoroarabino or a combination thereof, nucleotide sugar chemistry. Those skilled in the art will recognize that the foregoing are non-limiting examples and that any combination

of phosphate, sugar and base chemistry of a nucleic acid that supports the activity of RNase H enzyme is within the scope of the definition of the RNase H activating region and the instant invention.

By "2-5A antisense" or "2-5A antisense chimera" is meant an antisense oligonucleotide containing a 5'-phosphorylated 2'-5'-linked adenylate residue. These chimeras bind to target RNA in a sequence-specific manner and activate a cellular 2-5A-dependent ribonuclease which, in turn, cleaves the target RNA (Torrence et al., 1993 Proc. Natl. Acad. Sci. USA 90, 1300; Silverman et al., 2000, Methods Enzymol., 313, 522-533; Player and Torrence, 1998, Pharmacol. Ther., 78, 55-113).

By "triplex nucleic acid" or "triplex oligonucleotide" it is meant a polynucleotide or oligonucleotide that can bind to a double-stranded DNA in a sequence-specific manner to form a triple-strand helix. Formation of such triple helix structure has been shown to modulate transcription of the targeted gene (Duval-Valentin *et al.,* 1992, *Proc. Natl. Acad. Sci.USA,* 89, 504). Triplex nucleic acid molecules of the invention also include steric blocker nucleic acid molecules that bind to the Enhancer I region of HBV DNA (plus strand and/or minus strand) and prevent translation of HBV genomic DNA.

The term "single stranded RNA" (ssRNA) as used herein refers to a naturally occurring or synthetic ribonucleic acid molecule comprising a linear single strand, for example a ssRNA can be a messenger RNA (mRNA), transfer RNA (tRNA), ribosomal RNA (rRNA) etc. of a gene.

The term "single stranded DNA" (ssDNA) as used herein refers to a naturally occurring or synthetic deoxyribonucleic acid molecule comprising a linear single strand, for example, a ssDNA can be a sense or antisense gene sequence or EST (Expressed Sequence Tag).

The term "allozyme" as used herein refers to an allosteric enzymatic nucleic acid molecule, see for example George *et al.,* US Patent Nos. 5,834,186 and 5,741,679, Shih *et al.,* US Patent No. 5,589,332, Nathan *et al.,* US Patent No 5,871,914, Nathan and Ellington, International PCT publication No. WO 00/24931, Breaker *et al.,* International PCT Publication Nos. WO 00/26226 and 98/27104, and Sullenger *et al.,* International PCT publication No. WO 99/29842.

The term "2-5A chimera" as used herein refers to an oligonucleotide containing a 5'-phosphorylated 2'-5'-linked adenylate residue. These chimeras bind to target RNA in a sequence-specific manner and activate a cellular 2-5A-dependent ribonuclease which, in turn, cleaves the target RNA (Torrence *et al.,* 1993 *Proc. Natl. Acad. Sci. USA* 90, 1300;

Silverman *et al.*, 2000, *Methods Enzymol.*, 313, 522-533; Player and Torrence, 1998, *Pharmacol. Ther.*, 78, 55-113).

The term "double stranded RNA" or "dsRNA" as used herein refers to a double stranded RNA molecule capable of RNA interference "RNAi", including short interfering RNA "siRNA" see for example Bass, 2001, *Nature*, 411, 428-429; Elbashir et al., 2001, *Nature*, 411, 494-498; and Kreutzer *et al.*, International PCT Publication No. WO 00/44895; Zernicka-Goetz *et al.*, International PCT Publication No. WO 01/36646; Fire, International PCT Publication No. WO 99/32619; Plaetinck *et al.*, International PCT Publication No. WO 00/01846; Mello and Fire, International PCT Publication No. WO 01/29058; Deschamps-Depaillette, International PCT Publication No. WO 99/07409; and Li *et al.*, International PCT Publication No. WO 00/44914.

By "gene" it is meant, a nucleic acid that encodes an RNA, for example, nucleic acid sequences including, but not limited to, structural genes encoding a polypeptide.

By "complementarity" is meant that a nucleic acid can form hydrogen bond(s) with another nucleic acid sequence by either traditional Watson-Crick or other non-traditional types. In reference to the nucleic molecules of the present invention, the binding free energy for a nucleic acid molecule with its target or complementary sequence is sufficient to allow the relevant function of the nucleic acid to proceed, e.g., ribozyme cleavage, antisense or triple helix modulation. Determination of binding free energies for nucleic acid molecules is well known in the art (see, e.g., Turner et al., 1987, *CSH Symp. Quant. Biol.* LII pp.123-133; Frier et al., 1986, *Proc. Nat. Acad. Sci.* USA 83:9373-9377; Turner et al., 1987, *J. Am. Chem. Soc.* 109:3783-3785). A percent complementarity indicates the percentage of contiguous residues in a nucleic acid molecule that can form hydrogen bonds (e.g., Watson-Crick base pairing) with a second nucleic acid sequence (e.g., 5, 6, 7, 8, 9, 10 out of 10 being 50%, 60%, 70%, 80%, 90%, and 100% complementary). "Perfectly complementary" means that all the contiguous residues of a nucleic acid sequence will hydrogen bond with the same number of contiguous residues in a second nucleic acid sequence.

As used herein "cell" is used in its usual biological sense, and does not refer to an entire multicellular organism, e.g., specifically does not refer to a human. The cell can be present in an organism, e.g., birds, plants and mammals such as humans, cows, sheep, apes, monkeys, swine, dogs, and cats. The cell can be prokaryotic (e.g., bacterial cell) or eukaryotic (e.g., mammalian or plant cell).

By "HBV proteins" or "HCV proteins" is meant, a protein or a mutant protein derivative thereof, comprising sequence expressed and/or encoded by the HBV genome.

By "highly conserved sequence region" is meant a nucleotide sequence of one or more regions in a target gene does not vary significantly from one generation to the other or from one biological system to the other.

By "highly conserved nucleic acid binding region" is meant an amino acid sequence of one or more regions in a target protein that does not vary significantly from one generation to the other or from one biological system to the other.

By "related to the levels of HBV" is meant that the reduction of HBV expression (specifically HBV gene) RNA levels and thus reduction in the level of the respective protein will relieve, to some extent, the symptoms of the disease or condition.

By "related to the levels of HCV" is meant that the reduction of HCV expression (specifically HCV gene) RNA levels and thus reduction in the level of the respective protein will relieve, to some extent, the symptoms of the disease or condition.

By "RNA" is meant a molecule comprising at least one ribonucleotide residue. By "ribonucleotide" is meant a nucleotide with a hydroxyl group at the 2' position of a β-D-ribofuranose moiety.

By "vector" is meant any nucleic acid- and/or viral-based technique used to express and/or deliver a desired nucleic acid.

By "patient" is meant an organism, which is a donor or recipient of explanted cells or the cells themselves. "Patient" also refers to an organism to which the nucleic acid molecules of the invention can be administered. In one embodiment, a patient is a mammal or mammalian cells. In another embodiment, a patient is a human or human cells.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

First the drawings will be described briefly.

Drawings

Figure 1 shows the secondary structure model for seven different classes of enzymatic nucleic acid molecules. Arrow indicates the site of cleavage. --------- indicate the target sequence. Lines interspersed with dots are meant to indicate tertiary interactions. - is meant to

indicate base-paired interaction. **Group I Intron**: P1-P9.0 represent various stem-loop structures (Cech *et al.*, 1994, *Nature Struc. Bio.*, 1, 273). **RNase P (M1RNA)**: EGS represents external guide sequence (Forster *et al.*, 1990, *Science*, 249, 783; Pace *et al.*, 1990, *J. Biol. Chem.*, 265, 3587). **Group II Intron:** 5'SS means 5' splice site; 3'SS means 3'-splice site; IBS means intron binding site; EBS means exon binding site (Pyle *et al.*, 1994, *Biochemistry*, 33, 2716). **VS RNA:** I-VI are meant to indicate six stem-loop structures; shaded regions are meant to indicate tertiary interaction (Collins, International PCT Publication No. WO 96/19577). **HDV Ribozyme:** I-IV are meant to indicate four stem-loop structures (Been *et al.*, US Patent No. 5,625,047). **Hammerhead Ribozyme:** I-III are meant to indicate three stem-loop structures; stems I-III can be of any length and may be symmetrical or asymmetrical (Usman *et al.*, 1996, *Curr. Op. Struct. Bio.*, 1, 527). **Hairpin Ribozyme:** Helix 1, 4 and 5 can be of any length; Helix 2 is between 3 and 8 base-pairs long; Y is a pyrimidine; Helix 2 (H2) is provided with a least 4 base pairs (*i.e.*, n is 1, 2, 3 or 4) and helix 5 can be optionally provided of length 2 or more bases (preferably 3 - 20 bases, *i.e.*, m is from 1 - 20 or more). Helix 2 and helix 5 may be covalently linked by one or more bases (*i.e.*, r is $\geq$ 1 base). Helix 1, 4 or 5 may also be extended by 2 or more base pairs (*e.g.*, 4 - 20 base pairs) to stabilize the ribozyme structure, and preferably is a protein binding site. In each instance, each N and N' independently is any normal or modified base and each dash represents a potential base-pairing interaction. These nucleotides may be modified at the sugar, base or phosphate. Complete base-pairing is not required in the helices, but is preferred. Helix 1 and 4 can be of any size (*i.e.*, o and p is each independently from 0 to any number, *e.g.*, 20) as long as some base-pairing is maintained. Essential bases are shown as specific bases in the structure, but those in the art will recognize that one or more may be modified chemically (abasic, base, sugar and/or phosphate modifications) or replaced with another base without significant effect. Helix 4 can be formed from two separate molecules, *i.e.*, without a connecting loop. The connecting loop when present may be a ribonucleotide with or without modifications to its base, sugar or phosphate. "q" $\geq$ is 2 bases. The connecting loop can also be replaced with a non-nucleotide linker molecule. H refers to bases A, U, or C. Y refers to pyrimidine bases. "_____" refers to a covalent bond. (Burke *et al.*, 1996, *Nucleic Acids & Mol. Biol.*, 10, 129; Chowrira *et al.*, US Patent No. 5,631,359).

Figure 2 shows examples of chemically stabilized ribozyme motifs. **HH Rz**, represents hammerhead ribozyme motif (Usman *et al.*, 1996, *Curr. Op. Struct. Bio.*, 1, 527); **NCH Rz** represents the NCH ribozyme motif (Ludwig & Sproat, International PCT Publication No. WO 98/58058); **G-Cleaver**, represents G-cleaver ribozyme motif (Kore *et al.*, 1998, *Nucleic Acids Research*, 26, 4116-4120). **N** or **n**, represent independently a nucleotide which may be same or different and have complementarity to each other; **rI**, represents ribo-Inosine nucleotide; arrow indicates the site of cleavage within the target. Position 4 of the HH Rz and the NCH Rz is shown as having 2'-*C*-allyl modification, but

those skilled in the art will recognize that this position can be modified with other modifications well known in the art, so long as such modifications do not significantly inhibit the activity of the ribozyme.

Figure 3 shows an example of the Amberzyme ribozyme motif that is chemically stabilized (see, for example, Beigelman *et al.,* International PCT publication No. WO 99/55857; also referred to as Class I Motif). The Amberzyme motif is a class of enzymatic nucleic acid molecules that do not require the presence of a ribonucleotide (2'-OH) group for activity.

Figure 4 shows an example of the Zinzyme A ribozyme motif that is chemically stabilized (see, for example, International PCT publication No. WO 99/55857; also referred to as Class A Motif). The Zinzyme motif is a class of enzymatic nucleic acid molecules that do not require the presence of a ribonucleotide (2'-OH) group for activity.

Figure 5 shows an example of a DNAzyme motif described by Santoro *et al.,* 1997, *PNAS,* 94, 4262.

Figure 6 is a bar graph showing the percent change in serum HBV DNA levels following fourteen days of ribozyme treatment in HBV transgenic mice. Ribozymes targeting sites 273 (RPI.18341) and 1833 (RPI.18371) of HBV RNA administerd via continuous s.c. infusion at 10, 30, and 100 mg/kg/day are compared to continuous s.c. infusion administration of scrambled attenuated core ribozyme and saline controls, and orally administered 3TC® (300 mg/kg/day) and saline controls.

Figure 7 is a bar graph showing the mean serum HBV DNA levels following fourteen days of ribozyme treatment in HBV transgenic mice. Ribozymes targeting sites 273 (RPI.18341) and 1833 (RPI.18371) of HBV RNA administerd via continuous s.c. infusion at 10, 30, and 100 mg/kg/day are compared to continuous s.c. infusion administration of scrambled attenuated core ribozyme and saline controls, and orally administered 3TC® (300 mg/kg/day) and saline controls.

Figure 8 is a bar graph showing the decrease in serum HBV DNA (log) levels following fourteen days of ribozyme treatment in HBV transgenic mice. Ribozymes targeting sites 273 (RPI.18341) and 1833 (RPI.18371) of HBV RNA administerd via continuous s.c. infusion at 10, 30, and 100 mg/kg/day are compared to continuous s.c. infusion administration of scrambled attenuated core ribozyme and saline controls, and orally administered 3TC® (300 mg/kg/day) and saline controls.

Figure 9 is a bar graph showing the decrease in HBV DNA in HepG2.2.15 cells after treatment with ribozymes targeting sites 273 (RPI.18341), 1833 (RPI.18371), 1874

(RPI.18372), and 1873 (RPI.18418) of HBV RNA as compared to a scrambled attenuated core ribozyme (RPI.20995).

Figure 10 is a bar graph showing reduction in HBsAg levels following treatment of HepG2 cells with anti-HBV arm, stem, and loop-variant ribozymes (RPI.18341, RPI.22644, RPI.22645, RPI.22646, RPI.22647, RPI.22648, RPI.22649, and RPI.22650) targeting site 273 of the HBV pregenomic RNA as compared to a scrambled attenuated core ribozyme (RPI.20599).

Figure 11 is a bar graph showing reduction in HBsAg levels following treatment of HepG2 cells with RPI 18341 alone or in combination with Infergen®. At either 500 or 1000 units of Infergen®, the addition of 200 nM of RPI.18341 results in a 75-77% increase in anti-HBV activity as judged by the level of HBsAg secreted from the treated Hep G2 cells. Conversely, the anti-HBV activity of RPI.18341(at 200 nM) is increased 31-39% when used in combination of 500 or 1000 units of Infergen®.

Figure 12 is a bar graph showing reduction in HBsAg levels following treatment of HepG2 cells with RPI 18341 alone or in combination with Lamivudine. At 25 nM Lamivudine (3TC®), the addition of 100 nM of RPI.18341 results in a 48% increase in anti-HBV activity as judged by the level of HBsAg secreted from treated Hep G2 cells. Conversely, the anti-HBV activity of RPI.18341 (at 100 nM) is increased 31% when used in combination with 25 nM Lamivudine.

Figure 13 shows a scheme which outlines the steps involved in HBV reverse transcription. The HBV polymerase/reverse transcriptase binds to the 5'-stem-loop of the HBV pregenomic RNA and synthesizes a primer from the UUCA template. The reverse transcriptase and tetramer primer are translocated to the 3'-DR1 site. The RT primer binds to the UUCA sequence in the DR1 element and minus strand synthesis begins.

Figure 14 shows a non-limiting example of inhibition of HBV reverse transcription. A decoy molecule binds to the HBV RT primer, thereby preventing translocation of the RT to the 3'-DR1 site and preventing minus strand synthesis.

Figure 15 shows data of a HBV nucleic acid screen of 2'-O-allyl modified nucleic acid molecules. The levels of HbsAg were determined by ELISA. Inhibition of HBV is correlated to HBsAg antigen levels.

Figure 16 shows data of a HBV nucleic acid screen of 2'-O-methyl modified nucleic acid molecules. The levels of HbsAg were determined by ELISA. Inhibition of HBV is correlated to HBsAg antigen levels.

Figure 17 shows dose response data of 2'-O-methyl modified nucleic acid molecules targeting the HBV reverse transcriptase primer compared to levels of HBsAg.

Figure 18 shows data of nucleic acid screen of nucleic acid molecules (200 nM) targeting the HBV Enhancer I core region compared to levels of HBsAg.

Figure 19 shows data of nucleic acid screen of nucleic acid molecules (400 nM) targeting the HBV Enhancer I core region compared to levels of HBsAg.

Figure 20 shows dose response data of nucleic acid molecules targeting the HBV Enhancer I core region compared to levels of HBsAg.

Figure 21 shows a graph depicting HepG2.2.15 tumor growth in athymic nu/nu female mice as tumor volume (mm^3) vs time (days).

Figure 22 shows a graph depicting HepG2.2.15 tumor growth in athymic nu/nu female mice as tumor volume (mm^3) vs time (days). Inoculated HepG2.2.15 cells were selected for antibiotic resistance to G418 before introduction into the mouse.

Figure 23 is a schematic representation of the Dual Reporter System utilized to demonstrate enzymatic nucleic acid mediated reduction of luciferase activity in cell culture.

Figure 24 shows a schematic view of the secondary structure of the HCV 5'UTR (Brown *et al.*, 1992, *Nucleic Acids Res.*, 20, 5041-45; Honda *et al.*, 1999, *J. Virol.*, 73, 1165-74). Major structural domains are indicated in bold. Enzymatic nucleic acid cleavage sites are indicated by arrows. Solid arrows denote sites amenable to amino-modified enzymatic nucleic acid inhibition. Lead cleavage sites (195 and 330) are indicated with oversized solid arrows.

Figure 25 shows a non-limiting example of a nuclease resistant enzymatic nucleic acid molecule. Binding arms are indicated as stem I and stem III. Nucleotide modifications are indicated as follows: 2'-O-methyl nucleotides, lowercase; ribonucleotides, uppercase G, A; 2'-amino-uridine, u; inverted 3'-3' deoxyabasic, **B**. The positions of phosphorothioate linkages at the 5'-end of each enzymatic nucleic acid are indicated by subscript "s". *H* indicates A, C or U ribonucleotide, *N'* indicates A, C G or U ribonucleotide in substrate, *n* indicates base complementary to the N'. The U4 and U7 positions in the catalytic core are indicated.

Figure 26 is a set of bar graphs showing enzymatic nucleic acid mediated inhibition of HCV-luciferase expression in OST7 cells. OST7 cells were transfected with complexes containing reporter plasmids (2 μg/mL), enzymatic nucleic acids (100 nM) and lipid. The ratio of HCV-firefly luciferase luminescence/Renilla luciferase luminescence was determined

for each enzymatic nucleic acid tested and was compared to treatment with the ICR, an irrelevant control enzymatic nucleic acid lacking specificity to the HCV 5'UTR (adjusted to 1). Results are reported as the mean of triplicate samples ± SD. In **Figure 26A**, OST7 cells were treated with enzymatic nucleic acids (100 nM) targeting conserved sites (indicated by cleavage site) within the HCV 5'UTR. In **Figure 26B**, OST7 cells were treated with a subset of enzymatic nucleic acids to lead HCV sites (indicated by cleavage site) and corresponding attenuated core (AC) controls. Percent decrease in firefly/Renilla luciferase ratio after treatment with active enzymatic nucleic acids as compared to treatment with corresponding ACs is shown when the decrease is ≥ 50% and statistically significant. Similar results were obtained with 50 nM enzymatic nucleic acid.

Figure 27 is a series of line graphs showing the dose-dependent inhibition of HCV/luciferase expression following enzymatic nucleic acid treatment. Active enzymatic nucleic acid was mixed with corresponding AC to maintain a 100 nM total oligonucleotide concentration and the same lipid charge ratio. The concentration of active enzymatic nucleic acid for each point is shown. **Figure 27A–E** shows enzymatic nucleic acids targeting sites 79, 81, 142, 195, or 330, respectively. Results are reported as the mean of triplicate samples ± SD.

Figure 28 is a set of bar graphs showing reduction of HCV/luciferase RNA and inhibition of HCV-luciferase expression in OST7 cells. OST7 cells were transfected with complexes containing reporter plasmids (2 μg /ml), enzymatic nucleic acids, BACs or SACs (50 nM) and lipid. Results are reported as the mean of triplicate samples ± SD. In **Figure 28A** the ratio of HCV-firefly luciferase RNA/Renilla luciferase RNA is shown for each enzymatic nucleic acid or control tested. As compared to paired BAC controls (adjusted to 1), luciferase RNA levels were reduced by 40% and 25% for the site 195 or 330 enzymatic nucleic acids, respectively. In **Figure 28B** the ratio of HCV-firefly luciferase luminescence/Renilla luciferase luminescence is shown after treatment with site 195 or 330 enzymatic nucleic acids or paired controls. As compared to paired BAC controls (adjusted to 1), inhibition of protein expression was 70% and 40% for the site 195 or 330 enzymatic nucleic acids, respectively $P < 0.01$.

Figure 29 is a set a bar graphs showing interferon (IFN) alpha 2a and 2b dose response in combination with site 195 anti-HCV enzymatic nucleic acid treatment. **Figure 29A** shows data for IFN alfa 2a treatment. **Figure 29B** shows data for IFN alfa 2b treatment. Viral yield is reported from HeLa cells pretreated with IFN in units/ml (U/ml) as indicated for 4 h prior to infection and then treated with either 200 nM control (SAC) or site 195 anti-HCV enzymatic nucleic acid (195 RZ) for 24 h after infection. Cells were infected with a MOI =

0.1 for 30 min and collected at 24 h post infection. Error bars represent the S.D. of the mean of triplicate determinations.

Figure 30 is a line graph showing site 195 anti-HCV enzymatic nucleic acid dose response in combination with interferon (IFN) alpha 2a and 2b pretreatment. Viral yield is reported from HeLa cells pretreated for 4 h with or without IFN and treated with doses of site 195 anti-HCV enzymatic nucleic acid (195 RZ) as indicated for 24 h after infection. Anti-HCV enzymatic nucleic acid was mixed with control oligonucleotide (SAC) to maintain a constant 200 nM total dose of nucleic acid for delivery. Cells were infected with a MOI = 0.1 for 30 min and collected at 24 h post infection. Error bars represent the S.D. of the mean of triplicate determinations.

Figure 31 is a set of bar graphs showing data from consensus interferon (CIFN)/enzymatic nucleic acid combination treatment. **Figure 31A** shows CIFN dose response with site 195 anti-HCV enzymatic nucleic acid treatment. Viral yield is reported from cells pretreated with CIFN in units/ml (U/ml) as indicated and treated with either 200 nM control (SAC) or site 195 anti-HCV enzymatic nucleic acid (195 RZ). **Figure 31B** shows site 195 anti-HCV enzymatic nucleic acid dose response with CIFN pretreatment. Viral yield is reported from cells pretreated with or without CIFN and treated with concentrations of site 195 anti-HCV enzymatic nucleic acid (195 RZ) as indicated. Anti-HCV enzymatic nucleic acid was mixed with control oligonucleotide (SAC) to maintain a constant 200 nM total dose of nucleic acid for delivery. Cells were infected with a MOI = 0.1 for 30 min. and collected at 24 h post infection. Error bars represent the S.D. of the mean of triplicate determinations.

Figure 32 is a bar graph showing enzymatic nucleic acid activity and enhanced antiviral effect of an anti-HCV enzymatic nucleic acid targeting site 195 used in combination with consensus interferon (CIFN). Viral yield is reported from cells treated as indicated. BAC, cells were treated with 200 nM BAC (binding attenuated control) for 24 h after infection; CIFN+BAC, cells were treated with 12.5 U/ml CIFN for 4 h prior to infection and with 200 nM BAC for 24 h after infection; 195 RZ, cells were treated with 200 nM site 195 anti-HCV enzymatic nucleic acid for 24 h after infection; CIFN + 195 RZ, cells were treated with 12.5 U/ml CIFN for 4 h prior to infection and with 200 nM site 195 anti-HCV enzymatic nucleic acid for 24 h after infection. Cells were infected with a MOI = 0.1 for 30 min. Error bars represent the S.D. of the mean of triplicate determinations.

Figure 33 is a bar graph showing inhibition of a HCV-PV chimera replication by treatment with zinzyme enzymatic nucleic acid molecules targeting different sites within the HCV 5'-UTR compared to a scrambled attenuated core control (SAC) zinzyme.

Figure 34 is a bar graph showing inhibition of a HCV-PV chimera replication by antisense nucleic acid molecules targeting conserved regions of the HCV 5'-UTR compared to scrambled antisense controls.

Figure 35 shows the structure of compounds (2-5A) utilized in the study. "X" denotes the position of oxygen (O) in analog I or sulfur (S) in thiophosphate (P=S) analog II. The 2-5A compounds were synthesized, deprotected and purified as described herein utilizing CPG support with 3'-inverted abasic nucleotide. For chain extension 5'-O-(4,4'-dimetoxytrityl)-3'-O-(tert-butyldimethylsilyl)-N6-benzoyladenosine-2-cyanoethyl-N,N-diisopropyl-phosphoramidite (Chem. Genes Corp., Waltham, MA) was employed. Introduction of a 5'-terminal phosphate (analog I) or thiophosphate (analog II) group was performed with "Chemical Phosphorylation Reagent" (Glen Research, Sterling , VA). Structures of the final compounds were confirmed by MALDI-TOF analysis.

Figure 36 is a bar graph showing ribozyme activity and enhanced antiviral effect. (A) Interferon/ribozyme combination treatment. (B) 2-5A/ribozyme combination treatment. HeLa cells seeded in 96-well plates (10,000 cells per well) were pretreated as indicated for 4 hours. For pretreatment, SAC (RPI 17894), RZ (RPI 13919), and 2-5A analog I (RPI 21096) (200 nM) were complexed with lipid cytofectin. Cells were then infected with HCV-PV at a multiplicity of infection of 0.1. Virus inoculum was replaced after 30 minutes with media containing 5% serum and 100 nM RZ or SAC as indicated, complexed with cytofectin RPI.9778. After 20 hours, cells were lysed by 3 freeze/thaw cycles and virus was quantified by plaque assay. Plaque forming units (PFU)/ml are shown as the mean of triplicate samples + SEM. The absolute amount of viral yield in treated cells varied from day to day, presumably due to day to day variations in cell plating and transfection complexation. None, normal media; IFN, 10 U/ml consensus interferon; SAC, scrambled arm attenuated core control (RPI 17894); RZ, anti-HCV ribozyme (RPI 13919); 2-5A, (RPI 21096).

Figure 37 is a graph showing the inhibition of viral replication with anti-HCV ribozyme (RPI 13919) or 2-5A (RPI 21096) treatment. HeLa cells were treated as described in **Figure 36** except that there was no pretreatment and 200 nM oligonucleotide was used for treatment. 2-5A P=S contains a 5'-terminal thiophosphate (RPI21095) (see **Figure 35**).

Figure 38 is a bar graph showing anti-HCV ribozyme in combination with 2-5A treatment. HeLa cells were treated as described in **Figure 37** except concentrations were co-varied as shown to maintain a constant 200 nM total oligonucleotide dose for transfection. Cells treated with 50 nM anti-HCV ribozyme (RPI 13919) (middle bars) were also treated with 150 nM SAC (RPI 17894) or 2-5A (RPI 21096); likewise, cells treated with 100 nM anti-HCV ribozyme (bars at right) were also treated with 100 nM SAC or 2-5A.

Mechanism of action of Nucleic Acid Molecules of the Invention

Decoy: Nucleic acid decoy molecules are mimetics of naturally occurring nucleic acid molecules or portions of naturally occurring nucleic acid molecules that can be used to modulate the function of a specific protein or a nucleic acid whose activity is dependant on interaction with the naturally occurring nucleic acid molecule. Decoys modulate the function of a target protein or nucleic acid by competing with authentic nucleic acid binding to the ligand of interest. Often, the nucleic acid decoy is a truncated version of a nucleic acid sequence that is recognized, for example by a particular protein, such as a transcription factor or polymerase. Decoys can be chemically modified to increase binding affinity to the target ligand as well as to increase the enzymatic and chemical stability of the decoy. In addition, bridging and non-bridging linkers can be introduced into the decoy sequence to provide additional binding affinity to the target ligand. Decoy molecules of the invention that bind to an HCV or HBV target, such as HBV reverse transcriptase or HBV reverse transcriptase primer, or an enhancer region of the HBV pregenomic RNA, for example the Enhancer I element, modulate the transcription of RNA to DNA and therefore modulate expression of the pregenomic RNA of the virus (see **Figures 13 and 14**).

Aptamer: Nucleic acid aptamers can be selected to specifically bind to a particular ligand of interest (see for example Gold *et al.*, US 5,567,588 and US 5,475,096, Gold *et al.*, 1995, *Annu. Rev. Biochem.*, 64, 763; Brody and Gold, 2000, *J. Biotechnol.*, 74, 5; Sun, 2000, *Curr. Opin. Mol. Ther.*, 2, 100; Kusser, 2000, *J. Biotechnol.*, 74, 27; Hermann and Patel, 2000, *Science*, 287, 820; and Jayasena, 1999, *Clinical Chemistry*, 45, 1628). For example, the use of in vitro selection can be applied to evolve nucleic acid aptamers with binding specificity for HBV RT and/or HBV RT primer. Nucleic acid aptamers can include chemical modifications and linkers as described herein. Aptamer molecules of the invention that bind to a reverse transcriptase or reverse transcriptase primer, such as HBV reverse transcriptase or HBV reverse transcriptase primer, modulate the transcription of RNA to DNA and therefore modulate expression of the pregenomic RNA of the virus.

Antisense: Antisense molecules can be modified or unmodified RNA, DNA, or mixed polymer oligonucleotides and primarily function by specifically binding to matching sequences resulting in modulation of peptide synthesis (Wu-Pong, Nov 1994, *BioPharm*, 20-33). The antisense oligonucleotide binds to target RNA by Watson Crick base-pairing and blocks gene expression by preventing ribosomal translation of the bound sequences either by steric blocking or by activating RNase H enzyme. Antisense molecules can also alter protein synthesis by interfering with RNA processing or transport from the nucleus into the cytoplasm (Mukhopadhyay & Roth, 1996, *Crit. Rev. in Oncogenesis* 7, 151-190).

In addition, binding of single stranded DNA to RNA may result in nuclease degradation of the heteroduplex (Wu-Pong, *supra*; Crooke, *supra*). To date, the only backbone modified DNA chemistry which will act as substrates for RNase H are phosphorothioates, phosphorodithioates, and borontrifluoridates. Recently, it has been reported that 2'-arabino and 2'-fluoro arabino- containing oligos can also activate RNase H activity.

A number of antisense molecules have been described that utilize novel configurations of chemically modified nucleotides, secondary structure, and/or RNase H substrate domains (Woolf *et al.*, International PCT Publication No. WO 98/13526; Thompson *et al.*, USSN 60/082,404 which was filed on April 20, 1998; Hartmann *et al.*, USSN 60/101,174 which was filed on September 21, 1998) all of these are incorporated by reference herein in their entirety.

Antisense DNA can be used to target RNA by means of DNA-RNA interactions, thereby activating RNase H, which digests the target RNA in the duplex. Antisense DNA can be chemically synthesized or can be expressed via the use of a single stranded DNA intracellular expression vector or the equivalent thereof.

Triplex Forming Oligonucleotides (TFO): Single stranded oligonucleotide can be designed to bind to genomic DNA in a sequence specific manner. TFOs can be comprised of pyrimidine-rich oligonucleotides which bind DNA helices through Hoogsteen Base-pairing (Wu-Pong, *supra*). In addition, TFOs can be chemically modified to increase binding affinity to target DNA sequences. The resulting triple helix composed of the DNA sense, DNA antisense, and TFO disrupts RNA synthesis by RNA polymerase. The TFO mechanism can result in gene expression or cell death since binding may be irreversible (Mukhopadhyay & Roth, *supra*)

2'-5' Oligoadenylates: The 2-5A system is an interferon-mediated mechanism for RNA degradation found in higher vertebrates (Mitra *et al.*, 1996, *Proc Nat Acad Sci USA* 93, 6780-6785). Two types of enzymes, 2-5A synthetase and RNase L, are required for RNA cleavage. The 2-5A synthetases require double stranded RNA to form 2'-5' oligoadenylates (2-5A). 2-5A then acts as an allosteric effector for utilizing RNase L, which has the ability to cleave single stranded RNA. The ability to form 2-5A structures with double stranded RNA makes this system particularly useful for modulation of viral replication.

(2'-5') oligoadenylate structures can be covalently linked to antisense molecules to form chimeric oligonucleotides capable of RNA cleavage (Torrence, *supra*). These molecules putatively bind and activate a 2-5A-dependent RNase, the oligonucleotide/enzyme complex then binds to a target RNA molecule which can then be cleaved by the RNase enzyme. The covalent attachment of 2'-5' oligoadenylate structures is not limited to

antisense applications, and can be further elaborated to include attachment to nucleic acid molecules of the instant invention.

RNA interference (RNAi): RNA interference refers to the process of sequence specific post transcriptional gene silencing in animals mediated by short interfering RNAs (siRNA) (Fire *et al.*, 1998, *Nature,* 391, 806). The corresponding process in plants is commonly referred to as post transcriptional gene silencing or RNA silencing and is also referred to as quelling in fungi. The process of post transcriptional gene silencing is thought to be an evolutionarily conserved cellular defense mechanism used to prevent the expression of foreign genes which is commonly shared by diverse flora and phyla (Fire *et al.*, 1999, *Trends Genet.*, 15, 358). Such protection from foreign gene expression may have evolved in response to the production of double stranded RNAs (dsRNA) derived from viral infection or the random integration of transposon elements into a host genome via a cellular response that specifically destroys homologous single stranded RNA or viral genomic RNA. The presence of dsRNA in cells triggers the RNAi response though a mechanism that has yet to be fully characterized. This mechanism appears to be different from the interferon response that results from dsRNA mediated activation of protein kinase PKR and 2',5'-oligoadenylate synthetase resulting in non-specific cleavage of mRNA by ribonuclease L.

The presence of long dsRNAs in cells stimulates the activity of a ribonuclease III enzyme referred to as dicer. Dicer is involved in the processing of the dsRNA into short pieces of dsRNA known as short interfering RNAs (siRNA) (Berstein *et al.*, 2001, *Nature,* 409, 363). Short interfering RNAs derived from dicer activity are typically about 21-23 nucleotides in length and comprise about 19 base pair duplexes. Dicer has also been implicated in the excision of 21 and 22 nucleotide small temporal RNAs (stRNA) from precursor RNA of conserved structure that are implicated in translational control (Hutvagner *et al.*, 2001, *Science,* 293, 834). The RNAi response also features an endonuclease complex containing a siRNA, commonly referred to as an RNA-induced silencing complex (RISC), which mediates cleavage of single stranded RNA having sequence homologous to the siRNA. Cleavage of the target RNA takes place in the middle of the region complementary to the guide sequence of the siRNA duplex (Elbashir *et al.*, 2001, *Genes Dev.*, 15, 188).

Short interfering RNA mediated RNAi has been studied in a variety of systems. Fire *et al.*, 1998, *Nature,* 391, 806, were the first to observe RNAi in *C. Elegans*. Wianny and Goetz, 1999, *Nature Cell Biol.*, 2, 70, describes RNAi mediated by dsRNA in mouse embryos. Hammond *et al.*, 2000, *Nature,* 404, 293, describe RNAi in *Drosophila* cells transfected with dsRNA. Elbashir *et al.*, 2001, *Nature,* 411, 494, describe RNAi induced by introduction of duplexes of synthetic 21-nucleotide RNAs in cultured mammalian cells including human embryonic kidney and HeLa cells. Recent work in Drosophila embryonic lysates has revealed certain requirements for siRNA length, structure, chemical composition,

and sequence that are essential to mediate efficient RNAi activity. These studies have shown that 21 nucleotide siRNA duplexes are most active when containing two nucleotide 3'-overhangs. Furthermore, substitution of one or both siRNA strands with 2'-deoxy or 2'-O-methyl nucleotides abolishes RNAi activity, whereas substitution of 3'-terminal siRNA nucleotides with deoxy nucleotides was shown to be tolerated. Mismatch sequences in the center of the siRNA duplex were also shown to abolish RNAi activity. In addition, these studies also indicate that the position of the cleavage site in the target RNA is defined by the 5'-end of the siRNA guide sequence rather than the 3'-end (Elbashir *et al.*, 2001, *EMBO J.*, 20, 6877). Other studies have indicated that a 5'-phosphate on the target-complementary strand of a siRNA duplex is required for siRNA activity and that ATP is utilized to maintain the 5'-phosphate moiety on the siRNA (Nykanen *et al.*, 2001, *Cell,* 107, 309), however siRNA molecules lacking a 5'-phosphate are active when introduced exogenously, suggesting that 5'-phosphorylation of siRNA constructs may occur *in vivo*.

Enzymatic Nucleic Acid: Several varieties of naturally occurring enzymatic RNAs are presently known (Doherty and Doudna, 2001, *Annu. Rev. Biophys. Biomol. Struct.*, 30, 457-475; Symons, 1994, *Curr. Opin. Struct. Biol.*, 4, 322-30). In addition, several *in vitro* selection (evolution) strategies (Orgel, 1979, *Proc. R. Soc. London,* B 205, 435) have been used to evolve new nucleic acid catalysts capable of catalyzing cleavage and ligation of phosphodiester linkages (Joyce, 1989, *Gene,* 82, 83-87; Beaudry *et al.*, 1992, *Science* 257, 635-641; Joyce, 1992, *Scientific American* 267, 90-97; Breaker *et al.*, 1994, *TIBTECH* 12, 268; Bartel *et al.*, 1993, *Science* 261:1411-1418; Szostak, 1993, *TIBS* 17, 89-93; Kumar *et al.*, 1995, *FASEB J.*, 9, 1183; Breaker, 1996, *Curr. Op. Biotech.*, 7, 442; Santoro *et al.*, 1997, *Proc. Natl. Acad. Sci.*, 94, 4262; Tang *et al.*, 1997, *RNA* 3, 914; Nakamaye & Eckstein, 1994, *supra*; Long & Uhlenbeck, 1994, supra; Ishizaka et al., 1995, *supra*; Vaish *et al.*, 1997, *Biochemistry* 36, 6495). Each can catalyze a series of reactions including the hydrolysis of phosphodiester bonds in *trans* (and thus can cleave other RNA molecules) under physiological conditions.

Nucleic acid molecules of this invention can block HBV or HCV protein expression and can be used to treat disease or diagnose disease associated with the levels of HBV or HCV.

The enzymatic nature of an enzymatic nucleic acid has significant advantages, such as the concentration of nucleic acid necessary to affect a therapeutic treatment is low. This advantage reflects the ability of the enzymatic nucleic acid molecule to act enzymatically. Thus, a single enzymatic nucleic acid molecule is able to cleave many molecules of target RNA. In addition, the enzymatic nucleic acid molecule is a highly specific modulator, with the specificity of modulation depending not only on the base-pairing mechanism of binding to the target RNA, but also on the mechanism of target RNA cleavage. Single mismatches,

or base-substitutions, near the site of cleavage can be chosen to completely eliminate catalytic activity of an enzymatic nucleic acid molecule.

Nucleic acid molecules having an endonuclease enzymatic activity are able to repeatedly cleave other separate RNA molecules in a nucleotide base sequence-specific manner. With proper design and construction, such enzymatic nucleic acid molecules can be targeted to any RNA transcript, and efficient cleavage achieved *in vitro* (Zaug *et al.*, 324, *Nature* 429 1986; Uhlenbeck, 1987 *Nature* 328, 596; Kim et al., 84 *Proc. Natl. Acad. Sci. USA* 8788, 1987; Dreyfus, 1988, *Einstein Quart. J. Bio. Med.*, 6, 92; Haseloff and Gerlach, 334 *Nature* 585, 1988; Cech, 260 *JAMA* 3030, 1988; and Jefferies et al., 17 *Nucleic Acids Research* 1371, 1989; Chartrand *et al.*, 1995, *Nucleic Acids Research* 23, 4092; Santoro *et al.*, 1997, *PNAS* 94, 4262).

Because of their sequence specificity, *trans*-cleaving enzymatic nucleic acid molecules show promise as therapeutic agents for human disease (Usman & McSwiggen, 1995 *Ann. Rep. Med. Chem.* 30, 285-294; Christoffersen and Marr, 1995 *J. Med. Chem.* 38, 2023-2037). Enzymatic nucleic acid molecule can be designed to cleave specific RNA targets within the background of cellular RNA. Such a cleavage event renders the RNA non-functional and abrogates protein expression from that RNA. In this manner, synthesis of a protein associated with a disease state can be selectively modulated(Warashina *et al.*, 1999, *Chemistry and Biology*, 6, 237-250.

The present invention also features nucleic acid sensor molecules or allozymes having sensor domains comprising nucleic acid decoys and/or aptamers of the invention. Interaction of the nucleic acid sensor molecule's sensor domain with a molecular target, such as HCV or HBV target, e.g., HBV RT and/or HBV RT primer, can activate or inactivate the enzymatic nucleic acid domain of the nucleic acid sensor molecule, such that the activity of the nucleic acid sensor molecule is modulated in the presence of the target-signaling molecule. The nucleic acid sensor molecule can be designed to be active in the presence of the target molecule or alternately, can be designed to be inactive in the presence of the molecular target. For example, a nucleic acid sensor molecule is designed with a sensor domain having the sequence $(UUCA)_n$, where n is an integer from 1-10. In a non-limiting example, interaction of the HBV RT primer with the sensor domain of the nucleic acid sensor molecule can activate the enzymatic nucleic acid domain of the nucleic acid sensor molecule such that the sensor molecule catalyzes a reaction, for example cleavage of HBV RNA. In this example, the nucleic acid sensor molecule is activated in the presence of HBV RT or HBV RT primer, and can be used as a therapeutic to treat HBV infection. Alternately, the reaction can comprise cleavage or ligation of a labeled nucleic acid reporter molecule, providing a useful diagnostic reagent to detect the presence of HBV in a system.

HCV Target sites

Targets for useful nucleic acid molecules and nuclease activating compounds or chimeras can be determined as disclosed in Draper *et al.*, WO 93/23569; Sullivan *et al.*, WO 93/23057; Thompson *et al.*, WO 94/02595; Draper *et al.*, WO 95/04818; McSwiggen *et al.*, US Patent No. 5,525,468. Rather than repeat the guidance provided in those documents here, below are provided specific examples of such methods, not limiting to those in the art. Nucleic acid molecules and nuclease activating compounds or chimeras to such targets are designed as described in those applications and synthesized to be tested *in vitro* and *in vivo*, as also described. Such nucleic acid molecules and nuclease activating compounds or chimeras can also be optimized and delivered as described therein.

The sequence of HCV RNAs were screened for optimal enzymatic nucleic acid molecule target sites using a computer folding algorithm. Enzymatic nucleic acid cleavage sites were identified. These sites are shown in **Tables XVIII, XIX, XX and XXIII** (All sequences are 5' to 3' in the tables). The nucleotide base position is noted in the tables as that site to be cleaved by the designated type of enzymatic nucleic acid molecule. The nucleotide base position is noted in the tables as that site to be cleaved by the designated type of enzymatic nucleic acid molecule.

Because HCV RNAs are highly homologous in certain regions, some enzymatic nucleic acid molecule target sites are also homologous. In this case, a single enzymatic nucleic acid molecule will target different classes of HCV RNA. The advantage of one enzymatic nucleic acid molecule that targets several classes of HCV RNA is clear, especially in cases where one or more of these RNAs can contribute to the disease state.

Enzymatic nucleic acid molecules were designed that could bind and were individually analyzed by computer folding (Jaeger *et al.*, 1989 *Proc. Natl. Acad. Sci. USA*, 86, 7706) to assess whether the enzymatic nucleic acid molecule sequences fold into the appropriate secondary structure. Those enzymatic nucleic acid molecules with unfavorable intramolecular interactions between the binding arms and the catalytic core are eliminated from consideration. Varying binding arm lengths can be chosen to optimize activity. Generally, at least 5 bases on each arm are able to bind to, or otherwise interact with, the target RNA. Enzymatic nucleic acid molecules were designed to anneal to various sites in the mRNA message. The binding arms are complementary to the target site sequences described above.

HBV Target sites

Targets for useful ribozymes and antisense nucleic acids targeting HBV can be determined as disclosed in Draper *et al.*, WO 93/23569; Sullivan *et al.*, WO 93/23057; Thompson *et al.*, WO 94/02595; Draper *et al.*, WO 95/04818; McSwiggen *et al.*, US Patent No. 5,525,468. Other examples include the following PCT applications, which concern inactivation of expression of disease-related genes: WO 95/23225, WO 95/13380, WO 94/02595. Rather than repeat the guidance provided in those documents here, below are provided specific examples of such methods, not limiting to those in the art. Ribozymes and antisense to such targets are designed as described in those applications and synthesized to be tested *in vitro* and *in vivo*, as also described. The sequence of human HBV RNAs (for example, accession AF100308.1; HBV strain 2-18; additionally, other HBV strains can be screened by one skilled in the art, see **Table III** for other possible strains) were screened for optimal enzymatic nucleic acid and antisense target sites using a computer-folding algorithm. Antisense, hammerhead, DNAzyme, NCH (Inozyme), amberzyme, zinzyme or G-Cleaver ribozyme binding/cleavage sites were identified. These sites are shown in **Tables V to XI** (all sequences are 5' to 3' in the tables; X can be any base-paired sequence, the actual sequence is not relevant here). The nucleotide base position is noted in the Tables as that site to be cleaved by the designated type of enzymatic nucleic acid molecule. **Table IV** shows substrate positions selected from Renbo *et al.*, 1987, *Sci. Sin.*, 30, 507, used in Draper, USSN (07/882,712), filed May 14, 1992, entitled "METHOD AND REAGENT FOR INHIBITING HEPATITIS B VIRUS REPLICATION" and Draper et al., International PCT publication No. WO 93/23569, filed April 29, 1993, entitled "METHOD AND REAGENT FOR INHIBITING VIRAL REPLICATION". While human sequences can be screened and enzymatic nucleic acid molecule and/or antisense thereafter designed, as discussed in Stinchcomb *et al.*, WO 95/23225, mouse targeted ribozymes can be useful to test efficacy of action of the enzymatic nucleic acid molecule and/or antisense prior to testing in humans.

Antisense, hammerhead, DNAzyme, NCH (Inozyme), amberzyme, zinzyme or G-Cleaver ribozyme binding/cleavage sites were identified, as discussed above. The nucleic acid molecules were individually analyzed by computer folding (Jaeger *et al.*, 1989 *Proc. Natl. Acad. Sci. USA*, 86, 7706) to assess whether the sequences fold into the appropriate secondary structure. Those nucleic acid molecules with unfavorable intramolecular interactions such as between the binding arms and the catalytic core were eliminated from consideration. Varying binding arm lengths can be chosen to optimize activity.

Antisense, hammerhead, DNAzyme, NCH, amberzyme, zinzyme or G-Cleaver ribozyme binding/cleavage sites were identified and were designed to anneal to various sites in the RNA target. The binding arms are complementary to the target site sequences

described above. The nucleic acid molecules were chemically synthesized. The method of synthesis used follows the procedure for normal DNA/RNA synthesis as described below and in Usman *et al.*, 1987 *J. Am. Chem. Soc.*, 109, 7845; Scaringe *et al.*, 1990 *Nucleic Acids Res.*, 18, 5433; Wincott *et al.*, 1995 *Nucleic Acids Res.* 23, 2677-2684; and Caruthers *et al.*, 1992, *Methods in Enzymology* 211,3-19.

Synthesis of Nucleic acid Molecules

Synthesis of nucleic acids greater than 100 nucleotides in length is difficult using automated methods, and the therapeutic cost of such molecules is prohibitive. In this invention, small nucleic acid motifs ("small" refers to nucleic acid motifs no more than 100 nucleotides in length, preferably no more than 80 nucleotides in length, and most preferably no more than 50 nucleotides in length; *e.g.*, decoy nucleic acid molecules, aptamer nucleic acid molecules antisense nucleic acid molecules, enzymatic nucleic acid molecules) are preferably used for exogenous delivery. The simple structure of these molecules increases the ability of the nucleic acid to invade targeted regions of protein and/or RNA structure. Exemplary molecules of the instant invention are chemically synthesized, and others can similarly be synthesized.

Oligonucleotides (e.g., DNA oligonucleotides) are synthesized using protocols known in the art, for example as described in Caruthers *et al.*, 1992, *Methods in Enzymology* 211, 3-19, Thompson *et al.*, International PCT Publication No. WO 99/54459, Wincott *et al.*, 1995, *Nucleic Acids Res.* 23, 2677-2684, Wincott *et al.*, 1997, *Methods Mol. Bio.*, 74, 59, Brennan *et al.*, 1998, *Biotechnol Bioeng.*, 61, 33-45, and Brennan, US patent No. 6,001,311. The synthesis of oligonucleotides makes use of common nucleic acid protecting and coupling groups, such as dimethoxytrityl at the 5'-end, and phosphoramidites at the 3'-end. In a non-limiting example, small scale syntheses are conducted on a 394 Applied Biosystems, Inc. synthesizer using a 0.2 μmol scale protocol with a 2.5 min coupling step for 2'-O-methylated nucleotides and a 45 sec coupling step for 2'-deoxy nucleotides. **Table II** outlines the amounts and the contact times of the reagents used in the synthesis cycle. Alternatively, syntheses at the 0.2 μmol scale can be performed on a 96-well plate synthesizer, such as the instrument produced by Protogene (Palo Alto, CA) with minimal modification to the cycle. A 33-fold excess (60 μL of 0.11 M = 6.6 μmol) of 2'-O-methyl phosphoramidite and a 105-fold excess of S-ethyl tetrazole (60 μL of 0.25 M = 15 μmol) can be used in each coupling cycle of 2'-O-methyl residues relative to polymer-bound 5'-hydroxyl. A 22-fold excess (40 μL of 0.11 M = 4.4 μmol) of deoxy phosphoramidite and a 70-fold excess of S-ethyl tetrazole (40 μL of 0.25 M = 10 μmol) can be used in each coupling cycle of deoxy residues relative to polymer-bound 5'-hydroxyl. Average coupling yields on the 394 Applied Biosystems, Inc. synthesizer, determined by colorimetric quantitation of the trityl fractions, are typically 97.5-

99%. Other oligonucleotide synthesis reagents for the 394 Applied Biosystems, Inc. synthesizer include the following: detritylation solution is 3% TCA in methylene chloride (ABI); capping is performed with 16% *N*-methyl imidazole in THF (ABI) and 10% acetic anhydride/10% 2,6-lutidine in THF (ABI); and oxidation solution is 16.9 mM I_2, 49 mM pyridine, 9% water in THF (PERSEPTIVE™). Burdick & Jackson Synthesis Grade acetonitrile is used directly from the reagent bottle. S-Ethyltetrazole solution (0.25 M in acetonitrile) is made up from the solid obtained from American International Chemical, Inc. Alternately, for the introduction of phosphorothioate linkages, Beaucage reagent (3H-1,2-Benzodithiol-3-one 1,1-dioxide, 0.05 M in acetonitrile) is used.

Deprotection of the DNA-based oligonucleotides is performed as follows: the polymer-bound trityl-on oligoribonucleotide is transferred to a 4 mL glass screw top vial and suspended in a solution of 40% aq. methylamine (1 mL) at 65 °C for 10 min. After cooling to –20 °C, the supernatant is removed from the polymer support. The support is washed three times with 1.0 mL of EtOH:MeCN:H2O/3:1:1, vortexed and the supernatant is then added to the first supernatant. The combined supernatants, containing the oligoribonucleotide, are dried to a white powder.

The method of synthesis used for normal RNA including certain decoy nucleic acid molecules and enzymatic nucleic acid molecules follows the procedure as described in Usman *et al.*, 1987, *J. Am. Chem. Soc.*, 109, 7845; Scaringe *et al.*, 1990, *Nucleic Acids Res.*, 18, 5433; and Wincott *et al.*, 1995, *Nucleic Acids Res.* 23, 2677-2684 Wincott *et al.*, 1997, *Methods Mol. Bio.*, 74, 59, and makes use of common nucleic acid protecting and coupling groups, such as dimethoxytrityl at the 5'-end, and phosphoramidites at the 3'-end. In a non-limiting example, small scale syntheses are conducted on a 394 Applied Biosystems, Inc. synthesizer using a 0.2 μmol scale protocol with a 7.5 min coupling step for alkylsilyl protected nucleotides and a 2.5 min coupling step for 2'-O-methylated nucleotides. **Table II** outlines the amounts and the contact times of the reagents used in the synthesis cycle. Alternatively, syntheses at the 0.2 μmol scale can be done on a 96-well plate synthesizer, such as the instrument produced by Protogene (Palo Alto, CA) with minimal modification to the cycle. A 33-fold excess (60 μL of 0.11 M = 6.6 μmol) of 2'-O-methyl phosphoramidite and a 75-fold excess of S-ethyl tetrazole (60 μL of 0.25 M = 15 μmol) can be used in each coupling cycle of 2'-O-methyl residues relative to polymer-bound 5'-hydroxyl. A 66-fold excess (120 μL of 0.11 M = 13.2 μmol) of alkylsilyl (ribo) protected phosphoramidite and a 150-fold excess of S-ethyl tetrazole (120 μL of 0.25 M = 30 μmol) can be used in each coupling cycle of ribo residues relative to polymer-bound 5'-hydroxyl. Average coupling yields on the 394 Applied Biosystems, Inc. synthesizer, determined by colorimetric quantitation of the trityl fractions, are typically 97.5-99%. Other oligonucleotide synthesis reagents for the 394 Applied Biosystems, Inc. synthesizer include the following: detritylation

solution is 3% TCA in methylene chloride (ABI); capping is performed with 16% *N*-methyl imidazole in THF (ABI) and 10% acetic anhydride/10% 2,6-lutidine in THF (ABI); oxidation solution is 16.9 mM I_2, 49 mM pyridine, 9% water in THF (PERSEPTIVE™). Burdick & Jackson Synthesis Grade acetonitrile is used directly from the reagent bottle. S-Ethyltetrazole solution (0.25 M in acetonitrile) is made up from the solid obtained from American International Chemical, Inc. Alternately, for the introduction of phosphorothioate linkages, Beaucage reagent (3H-1,2-Benzodithiol-3-one 1,1-dioxide0.05 M in acetonitrile) is used.

Deprotection of the RNA is performed using either a two-pot or one-pot protocol. For the two-pot protocol, the polymer-bound trityl-on oligoribonucleotide is transferred to a 4 mL glass screw top vial and suspended in a solution of 40% aq. methylamine (1 mL) at 65 °C for 10 min. After cooling to –20 °C, the supernatant is removed from the polymer support. The support is washed three times with 1.0 mL of EtOH:MeCN:H2O/3:1:1, vortexed and the supernatant is then added to the first supernatant. The combined supernatants, containing the oligoribonucleotide, are dried to a white powder. The base deprotected oligoribonucleotide is resuspended in anhydrous TEA/HF/NMP solution (300 µL of a solution of 1.5 mL N-methylpyrrolidinone, 750 µL TEA and 1 mL TEA•3HF to provide a 1.4 M HF concentration) and heated to 65 °C. After 1.5 h, the oligomer is quenched with 1.5 M NH_4HCO_3.

Alternatively, for the one-pot protocol, the polymer-bound trityl-on oligoribonucleotide is transferred to a 4 mL glass screw top vial and suspended in a solution of 33% ethanolic methylamine/DMSO: 1/1 (0.8 mL) at 65 °C for 15 min. The vial is brought to r.t. TEA•3HF (0.1 mL) is added and the vial is heated at 65 °C for 15 min. The sample is cooled at –20 °C and then quenched with 1.5 M NH_4HCO_3.

For purification of the trityl-on oligomers, the quenched NH_4HCO_3 solution is loaded onto a C-18 containing cartridge that had been prewashed with acetonitrile followed by 50 mM TEAA. After washing the loaded cartridge with water, the RNA is detritylated with 0.5% TFA for 13 min. The cartridge is then washed again with water, salt exchanged with 1 M NaCl and washed with water again. The oligonucleotide is then eluted with 30% acetonitrile.

Inactive hammerhead ribozymes or binding attenuated control (BAC) oligonucleotides are synthesized by substituting a U for G_5 and a U for A_{14} (numbering from Hertel, K. J., *et al.*, 1992, *Nucleic Acids Res.*, 20, 3252). Similarly, one or more nucleotide substitutions can be introduced in other nucleic acid decoy molecules to inactivate the molecule and such molecules can serve as a negative control.

The average stepwise coupling yields are typically >98% (Wincott *et al.*, 1995 *Nucleic Acids Res.* 23, 2677-2684). Those of ordinary skill in the art will recognize that the scale of synthesis can be adapted to be larger or smaller than the example described above including but not limited to 96-well format, all that is important is the ratio of chemicals used in the reaction.

Alternatively, the nucleic acid molecules of the present invention can be synthesized separately and joined together post-synthetically, for example, by ligation (Moore *et al.*, 1992, *Science* 256, 9923; Draper *et al.*, International PCT publication No. WO 93/23569; Shabarova *et al.*, 1991, *Nucleic Acids Research* 19, 4247; Bellon *et al.*, 1997, *Nucleosides & Nucleotides*, 16, 951; Bellon *et al.*, 1997, *Bioconjugate Chem.* 8, 204).

The nucleic acid molecules of the present invention can be modified extensively to enhance stability by modification with nuclease resistant groups, for example, 2'-amino, 2'-*C*-allyl, 2'-flouro, 2'-*O*-methyl, 2'-H (for a review see Usman and Cedergren, 1992, *TIBS* 17, 34; Usman *et al.*, 1994, *Nucleic Acids Symp. Ser.* 31, 163). Ribozymes can be purified by gel electrophoresis using general methods or can be purified by high pressure liquid chromatography (HPLC; see Wincott *et al.*, *supra*, the totality of which is hereby incorporated herein by reference) and re-suspended in water.

The sequences of the nucleic acid molecules that are chemically synthesized, useful in this study, are shown in **Tables XI, XV, XX, XXI, XXII and XXIII**. The nucleic acid sequences listed in **Tables IV-XI, XIV-XV and XVIII-XXIII** can be formed of ribonucleotides or other nucleotides or non-nucleotides. Such nucleic acid sequences are equivalent to the sequences described specifically in the Tables.

Optimizing Activity of the nucleic acid molecule of the invention

Chemically synthesizing nucleic acid molecules with modifications (base, sugar and/or phosphate) can prevent their degradation by serum ribonucleases, which can increase their potency (see *e.g.*, Eckstein *et al.*, International Publication No. WO 92/07065; Perrault *et al.*, 1990 *Nature* 344, 565; Pieken et al., 1991, *Science* 253, 314; Usman and Cedergren, 1992, *Trends in Biochem. Sci.* 17, 334; Usman *et al.*, International Publication No. WO 93/15187; and Rossi *et al.*, International Publication No. WO 91/03162; Sproat, US Patent No. 5,334,711; Gold *et al.*, US 6,300,074; and Burgin *et al.*, *supra;* all of which are incorporated by reference herein). All of the above references describe various chemical modifications that can be made to the base, phosphate and/or sugar moieties of the nucleic acid molecules described herein. Modifications that enhance their efficacy in cells, and removal of bases from nucleic acid molecules to shorten oligonucleotide synthesis times and reduce chemical requirements are desired.

There are several examples in the art describing sugar, base and phosphate modifications that can be introduced into nucleic acid molecules with significant enhancement in their nuclease stability and efficacy. For example, oligonucleotides are modified to enhance stability and/or enhance biological activity by modification with nuclease resistant groups, for example, 2'-amino, 2'-*C*-allyl, 2'-flouro, 2'-*O*-methyl, 2'-H, nucleotide base modifications (for a review see Usman and Cedergren, 1992, *TIBS.* 17, 34; Usman *et al.,* 1994, *Nucleic Acids Symp. Ser.* 31, 163; Burgin *et al.,* 1996, *Biochemistry,* 35, 14090). Sugar modification of nucleic acid molecules have been extensively described in the art (see Eckstein *et al., International Publication* PCT No. WO 92/07065; Perrault *et al. Nature,* 1990, *344,* 565-568; Pieken *et al. Science,* 1991, *253,* 314-317; Usman and Cedergren, *Trends in Biochem. Sci.* , 1992, *17,* 334-339; Usman *et al. International Publication* PCT No. WO 93/15187; Sproat, *US Patent* No. 5,334,711 and Beigelman *et al.,* 1995, *J. Biol. Chem.,* 270, 25702; Beigelman *et al.,* International PCT publication No. WO 97/26270; Beigelman *et al.,* US Patent No. 5,716,824; Usman *et al.,* US patent No. 5,627,053; Woolf *et al.,* International PCT Publication No. WO 98/13526; Thompson *et al.,* USSN 60/082,404 which was filed on April 20, 1998; Karpeisky *et al.,* 1998, *Tetrahedron Lett.,* 39, 1131; Earnshaw and Gait, 1998, *Biopolymers (Nucleic Acid Sciences),* 48, 39-55; Verma and Eckstein, 1998, *Annu. Rev. Biochem.,* 67, 99-134; and Burlina *et al.,* 1997, *Bioorg. Med. Chem.,* 5, 1999-2010; all of the references are hereby incorporated in their totality by reference herein). Such publications describe general methods and strategies to determine the location of incorporation of sugar, base and/or phosphate modifications and the like into ribozymes without modulating catalysis, and are incorporated by reference herein. In view of such teachings, similar modifications can be used as described herein to modify the nucleic acid molecules of the instant invention.

While chemical modification of oligonucleotide internucleotide linkages with phosphorothioate, phosphorothioate, and/or 5'-methylphosphonate linkages improves stability, excessive modifications can cause some toxicity. Therefore, when designing nucleic acid molecules, the amount of these internucleotide linkages should be minimized. The reduction in the concentration of these linkages should lower toxicity, resulting in increased efficacy and higher specificity of these molecules.

Nucleic acid molecules having chemical modifications that maintain or enhance activity are provided. Such a nucleic acid is also generally more resistant to nucleases than an unmodified nucleic acid. Accordingly, the *in vitro* and/or *in vivo* activity should not be significantly lowered. In cases in which modulation is the goal, therapeutic nucleic acid molecules delivered exogenously should optimally be stable within cells until translation of the target RNA has been modulated long enough to reduce the levels of the undesirable protein. This period of time varies between hours to days depending upon the disease state.

Improvements in the chemical synthesis of RNA and DNA (Wincott *et al.*, 1995 *Nucleic Acids Res.* 23, 2677; Caruthers *et al.*, 1992, *Methods in Enzymology* 211,3-19 (incorporated by reference herein)) have expanded the ability to modify nucleic acid molecules by introducing nucleotide modifications to enhance their nuclease stability, as described above.

In one embodiment, nucleic acid molecules of the invention include one or more G-clamp nucleotides. A G-clamp nucleotide is a modified cytosine analog wherein the modifications confer the ability to hydrogen bond both Watson-Crick and Hoogsteen faces of a complementary guanine within a duplex, see for example Lin and Matteucci, 1998, *J. Am. Chem. Soc.*, 120, 8531-8532. A single G-clamp analog substation within an oligonucleotide can result in substantially enhanced helical thermal stability and mismatch discrimination when hybridized to complementary oligonucleotides. The inclusion of such nucleotides in nucleic acid molecules of the invention results in both enhanced affinity and specificity to nucleic acid targets. In another embodiment, nucleic acid molecules of the invention include one or more LNA "locked nucleic acid" nucleotides such as a 2', 4'-C methylene bicyclo nucleotide (see for example Wengel *et al.*, International PCT Publication No. WO 00/66604 and WO 99/14226).

In another embodiment, the invention features conjugates and/or complexes of nucleic acid molecules targeting HBV or HCV. Such conjugates and/or complexes can be used to facilitate delivery of molecules into a biological system, such as a cell. The conjugates and complexes provided by the instant invention can impart therapeutic activity by transferring therapeutic compounds across cellular membranes, altering the pharmacokinetics, and/or modulating the localization of nucleic acid molecules of the invention. The present invention encompasses the design and synthesis of novel conjugates and complexes for the delivery of molecules, including, but not limited to, small molecules, lipids, phospholipids, nucleosides, nucleotides, nucleic acids, antibodies, toxins, negatively charged polymers and other polymers, for example proteins, peptides, hormones, carbohydrates, polyethylene glycols, or polyamines, across cellular membranes. In general, the transporters described are designed to be used either individually or as part of a multi-component system, with or without degradable linkers. These compounds are expected to improve delivery and/or localization of nucleic acid molecules of the invention into a number of cell types originating from different tissues, in the presence or absence of serum (see Sullenger and Cech, US 5,854,038). Conjugates of the molecules described herein can be attached to biologically active molecules via linkers that are biodegradable, such as biodegradable nucleic acid linker molecules.

The term "biodegradable nucleic acid linker molecule" as used herein, refers to a nucleic acid molecule that is designed as a biodegradable linker to connect one molecule to another molecule, for example, a biologically active molecule. The stability of the

biodegradable nucleic acid linker molecule can be modulated by using various combinations of ribonucleotides, deoxyribonucleotides, and chemically modified nucleotides, for example, 2'-O-methyl, 2'-fluoro, 2'-amino, 2'-O-amino, 2'-C-allyl, 2'-O-allyl, and other 2'-modified or base modified nucleotides. The biodegradable nucleic acid linker molecule can be a dimer, trimer, tetramer or longer nucleic acid molecule, for example, an oligonucleotide of about 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 nucleotides in length, or can comprise a single nucleotide with a phosphorus-based linkage, for example, a phosphoramidate or phosphodiester linkage. The biodegradable nucleic acid linker molecule can also comprise nucleic acid backbone, nucleic acid sugar, or nucleic acid base modifications.

The term "biodegradable" as used herein, refers to degradation in a biological system, for example enzymatic degradation or chemical degradation.

The term "biologically active molecule" as used herein, refers to compounds or molecules that are capable of eliciting or modifying a biological response in a system. Non-limiting examples of biologically active molecules contemplated by the instant invention include therapeutically active molecules such as antibodies, hormones, antivirals, peptides, proteins, chemotherapeutics, small molecules, vitamins, co-factors, nucleosides, nucleotides, oligonucleotides, enzymatic nucleic acids, antisense nucleic acids, triplex forming oligonucleotides, 2,5-A chimeras, siRNA, dsRNA, allozymes, aptamers, decoys and analogs thereof. Biologically active molecules of the invention also include molecules capable of modulating the pharmacokinetics and/or pharmacodynamics of other biologically active molecules, for example, lipids and polymers such as polyamines, polyamides, polyethylene glycol and other polyethers.

The term "phospholipid" as used herein, refers to a hydrophobic molecule comprising at least one phosphorus group. For example, a phospholipid can comprise a phosphorus-containing group and saturated or unsaturated alkyl group, optionally substituted with OH, COOH, oxo, amine, or substituted or unsubstituted aryl groups.

Therapeutic nucleic acid molecules (*e.g.*, decoy nucleic acid molecules) delivered exogenously optimally are stable within cells until reverse trascription of the pregenomic RNA has been modulated long enough to reduce the levels of HBV or HCV DNA. The nucleic acid molecules are resistant to nucleases in order to function as effective intracellular therapeutic agents. Improvements in the chemical synthesis of nucleic acid molecules described in the instant invention and in the art have expanded the ability to modify nucleic acid molecules by introducing nucleotide modifications to enhance their nuclease stability as described above.

In yet another embodiment, nucleic acid molecules having chemical modifications that maintain or enhance enzymatic activity are provided. Such nucleic acids are also generally more resistant to nucleases than unmodified nucleic acids. Thus, *in vitro* and/or *in vivo* the activity should not be significantly lowered. As exemplified herein, such nucleic acid molecules are useful *in vitro* and/or *in vivo* even if activity over all is reduced 10 fold (Burgin *et al.*, 1996, *Biochemistry*, 35, 14090).

Use of the nucleic acid-based molecules of the invention will lead to better treatment of the disease progression by affording the possibility of combination therapies (*e.g.*, multiple antisense, nucleic acid decoy, or nucleic acid aptamer molecules targeted to different genes; nucleic acid molecules coupled with known small molecule modulators ors; or intermittent treatment with combinations of molecules (including different motifs) and/or other chemical or biological molecules). The treatment of patients with nucleic acid molecules may also include combinations of different types of nucleic acid molecules.

In another aspect the nucleic acid molecules comprise a 5' and/or a 3'- cap structure.

By "cap structure" is meant chemical modifications, which have been incorporated at either terminus of the oligonucleotide (see, for example, Wincott *et al.*, WO 97/26270, incorporated by reference herein). These terminal modifications protect the nucleic acid molecule from exonuclease degradation, and may help in delivery and/or localization within a cell. The cap may be present at the 5'-terminus (5'-cap) or at the 3'-terminal (3'-cap) or may be present on both termini. In non-limiting examples: the 5'-cap is selected from the group comprising inverted abasic residue (moiety); 4',5'-methylene nucleotide; 1-(beta-D-erythrofuranosyl) nucleotide, 4'-thio nucleotide; carbocyclic nucleotide; 1,5-anhydrohexitol nucleotide; L-nucleotides; alpha-nucleotides; modified base nucleotide; phosphorodithioate linkage; *threo*-pentofuranosyl nucleotide; acyclic 3',4'-seco nucleotide; acyclic 3,4-dihydroxybutyl nucleotide; acyclic 3,5-dihydroxypentyl nucleotide, 3'-3'-inverted nucleotide moiety; 3'-3'-inverted abasic moiety; 3'-2'-inverted nucleotide moiety; 3'-2'-inverted abasic moiety; 1,4-butanediol phosphate; 3'-phosphoramidate; hexylphosphate; aminohexyl phosphate; 3'-phosphate; 3'-phosphorothioate; phosphorodithioate; or bridging or non-bridging methylphosphonate moiety (for more details, see Wincott *et al.*, International PCT publication No. WO 97/26270, incorporated by reference herein).

In yet another preferred embodiment, the 3'-cap is selected from a group comprising, 4',5'-methylene nucleotide; 1-(beta-D-erythrofuranosyl) nucleotide; 4'-thio nucleotide, carbocyclic nucleotide; 5'-amino-alkyl phosphate; 1,3-diamino-2-propyl phosphate; 3-aminopropyl phosphate; 6-aminohexyl phosphate; 1,2-aminododecyl phosphate; hydroxypropyl phosphate; 1,5-anhydrohexitol nucleotide; L-nucleotide; alpha-nucleotide; modified base nucleotide; phosphorodithioate; *threo*-pentofuranosyl nucleotide; acyclic 3',4'-

seco nucleotide; 3,4-dihydroxybutyl nucleotide; 3,5-dihydroxypentyl nucleotide, 5'-5'-inverted nucleotide moiety; 5'-5'-inverted abasic moiety; 5'-phosphoramidate; 5'-phosphorothioate; 1,4-butanediol phosphate; 5'-amino; bridging and/or non-bridging 5'-phosphoramidate, phosphorothioate and/or phosphorodithioate, bridging or non bridging methylphosphonate and 5'-mercapto moieties (for more details see Beaucage and Iyer, 1993, *Tetrahedron* 49, 1925; incorporated by reference herein).

By the term "non-nucleotide" is meant any group or compound which can be incorporated into a nucleic acid chain in the place of one or more nucleotide units, including either sugar and/or phosphate substitutions, and allows the remaining bases to exhibit their enzymatic activity. The group or compound is abasic in that it does not contain a commonly recognized nucleotide base, such as adenosine, guanine, cytosine, uracil or thymine.

The term "alkyl" as used herein refers to a saturated aliphatic hydrocarbon, including straight-chain, branched-chain "isoalkyl", and cyclic alkyl groups. The term "alkyl" also comprises alkoxy, alkyl-thio, alkyl-thio-alkyl, alkoxyalkyl, alkylamino, alkenyl, alkynyl, alkoxy, cycloalkenyl, cycloalkyl, cycloalkylalkyl, heterocycloalkyl, heteroaryl, C1-C6 hydrocarbyl, aryl or substituted aryl groups. Preferably, the alkyl group has 1 to 12 carbons. More preferably it is a lower alkyl of from about 1 to 7 carbons, more preferably about 1 to 4 carbons. The alkyl group can be substituted or unsubstituted. When substituted the substituted group(s) preferably comprise hydroxy, oxy, thio, amino, nitro, cyano, alkoxy, alkyl-thio, alkyl-thio-alkyl, alkoxyalkyl, alkylamino, silyl, alkenyl, alkynyl, alkoxy, cycloalkenyl, cycloalkyl, cycloalkylalkyl, heterocycloalkyl, heteroaryl, C1-C6 hydrocarbyl, aryl or substituted aryl groups. The term "alkyl" also includes alkenyl groups containing at least one carbon-carbon double bond, including straight-chain, branched-chain, and cyclic groups. Preferably, the alkenyl group has about 2 to 12 carbons. More preferably it is a lower alkenyl of from about 2 to 7 carbons, more preferably about 2 to 4 carbons. The alkenyl group can be substituted or unsubstituted. When substituted the substituted group(s) preferably comprise hydroxy, oxy, thio, amino, nitro, cyano, alkoxy, alkyl-thio, alkyl-thio-alkyl, alkoxyalkyl, alkylamino, silyl, alkenyl, alkynyl, alkoxy, cycloalkenyl, cycloalkyl, cycloalkylalkyl, heterocycloalkyl, heteroaryl, C1-C6 hydrocarbyl, aryl or substituted aryl groups. The term "alkyl" also includes alkynyl groups containing at least one carbon-carbon triple bond, including straight-chain, branched-chain, and cyclic groups. Preferably, the alkynyl group has about 2 to 12 carbons. More preferably it is a lower alkynyl of from about 2 to 7 carbons, more preferably about 2 to 4 carbons. The alkynyl group can be substituted or unsubstituted. When substituted the substituted group(s) preferably comprise hydroxy, oxy, thio, amino, nitro, cyano, alkoxy, alkyl-thio, alkyl-thio-alkyl, alkoxyalkyl, alkylamino, silyl, alkenyl, alkynyl, alkoxy, cycloalkenyl, cycloalkyl, cycloalkylalkyl, heterocycloalkyl, heteroaryl, C1-C6 hydrocarbyl, aryl or substituted aryl groups. Alkyl groups or moieties of

the invention can also include aryl, alkylaryl, carbocyclic aryl, heterocyclic aryl, amide and ester groups. The preferred substituent(s) of aryl groups are halogen, trihalomethyl, hydroxyl, SH, OH, cyano, alkoxy, alkyl, alkenyl, alkynyl, and amino groups. An "alkylaryl" group refers to an alkyl group (as described above) covalently joined to an aryl group (as described above). Carbocyclic aryl groups are groups wherein the ring atoms on the aromatic ring are all carbon atoms. The carbon atoms are optionally substituted. Heterocyclic aryl groups are groups having from about 1 to 3 heteroatoms as ring atoms in the aromatic ring and the remainder of the ring atoms are carbon atoms. Suitable heteroatoms include oxygen, sulfur, and nitrogen, and include furanyl, thienyl, pyridyl, pyrrolyl, N-lower alkyl pyrrolo, pyrimidyl, pyrazinyl, imidazolyl and the like, all optionally substituted. An "amide" refers to an -C(O)-NH-R, where R is either alkyl, aryl, alkylaryl or hydrogen. An "ester" refers to an -C(O)-OR', where R is either alkyl, aryl, alkylaryl or hydrogen.

The term "alkoxyalkyl" as used herein refers to an alkyl-O-alkyl ether, for example methoxyethyl or ethoxymethyl.

The term "alkyl-thio-alkyl" as used herein refers to an alkyl-S-alkyl thioether, for example methylthiomethyl or methylthioethyl.

The term "amination" as used herein refers to a process in which an amino group or substituted amine is introduced into an organic molecule.

The term "exocyclic amine protecting moiety" as used herein refers to a nucleobase amino protecting group compatible with oligonucleotide synthesis, for example an acyl or amide group.

The term "alkenyl" as used herein refers to a straight or branched hydrocarbon of a designed number of carbon atoms containing at least one carbon-carbon double bond. Examples of "alkenyl" include vinyl, allyl, and 2-methyl-3-heptene.

The term "alkoxy" as used herein refers to an alkyl group of indicated number of carbon atoms attached to the parent molecular moiety through an oxygen bridge. Examples of alkoxy groups include, for example, methoxy, ethoxy, propoxy and isopropoxy.

The term "alkynyl" as used herein refers to a straight or branched hydrocarbon of a designed number of carbon atoms containing at least one carbon-carbon triple bond. Examples of "alkynyl" include propargyl, propyne, and 3-hexyne.

The term "aryl" as used herein refers to an aromatic hydrocarbon ring system containing at least one aromatic ring. The aromatic ring can optionally be fused or otherwise attached to other aromatic hydrocarbon rings or non-aromatic hydrocarbon rings. Examples

of aryl groups include, for example, phenyl, naphthyl, 1,2,3,4-tetrahydronaphthalene and biphenyl. Preferred examples of aryl groups include phenyl and naphthyl.

The term "cycloalkenyl" as used herein refers to a C3-C8 cyclic hydrocarbon containing at least one carbon-carbon double bond. Examples of cycloalkenyl include cyclopropenyl, cyclobutenyl, cyclopentenyl, cyclopentadiene, cyclohexenyl, 1,3-cyclohexadiene, cycloheptenyl, cycloheptatrienyl, and cyclooctenyl.

The term "cycloalkyl" as used herein refers to a C3-C8 cyclic hydrocarbon. Examples of cycloalkyl include cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, cycloheptyl and cyclooctyl.

The term "cycloalkylalkyl," as used herein, refers to a C3-C7 cycloalkyl group attached to the parent molecular moiety through an alkyl group, as defined above. Examples of cycloalkylalkyl groups include cyclopropylmethyl and cyclopentylethyl.

The terms "halogen" or "halo" as used herein refers to indicate fluorine, chlorine, bromine, and iodine.

The term "heterocycloalkyl," as used herein refers to a non-aromatic ring system containing at least one heteroatom selected from nitrogen, oxygen, and sulfur. The heterocycloalkyl ring can be optionally fused to or otherwise attached to other heterocycloalkyl rings and/or non-aromatic hydrocarbon rings. Preferred heterocycloalkyl groups have from 3 to 7 members. Examples of heterocycloalkyl groups include, for example, piperazine, morpholine, piperidine, tetrahydrofuran, pyrrolidine, and pyrazole. Preferred heterocycloalkyl groups include piperidinyl, piperazinyl, morpholinyl, and pyrolidinyl.

The term "heteroaryl" as used herein refers to an aromatic ring system containing at least one heteroatom selected from nitrogen, oxygen, and sulfur. The heteroaryl ring can be fused or otherwise attached to one or more heteroaryl rings, aromatic or non-aromatic hydrocarbon rings or heterocycloalkyl rings. Examples of heteroaryl groups include, for example, pyridine, furan, thiophene, 5,6,7,8-tetrahydroisoquinoline and pyrimidine. Preferred examples of heteroaryl groups include thienyl, benzothienyl, pyridyl, quinolyl, pyrazinyl, pyrimidyl, imidazolyl, benzimidazolyl, furanyl, benzofuranyl, thiazolyl, benzothiazolyl, isoxazolyl, oxadiazolyl, isothiazolyl, benzisothiazolyl, triazolyl, tetrazolyl, pyrrolyl, indolyl, pyrazolyl, and benzopyrazolyl.

The term "C1-C6 hydrocarbyl" as used herein refers to straight, branched, or cyclic alkyl groups having 1-6 carbon atoms, optionally containing one or more carbon-carbon double or triple bonds. Examples of hydrocarbyl groups include, for example, methyl, ethyl,

propyl, isopropyl, n-butyl, sec-butyl, tert-butyl, pentyl, 2-pentyl, isopentyl, neopentyl, hexyl, 2-hexyl, 3-hexyl, 3-methylpentyl, vinyl, 2-pentene, cyclopropylmethyl, cyclopropyl, cyclohexylmethyl, cyclohexyl and propargyl. When reference is made herein to C1-C6 hydrocarbyl containing one or two double or triple bonds it is understood that at least two carbons are present in the alkyl for one double or triple bond, and at least four carbons for two double or triple bonds.

The term "nucleotide" as used herein refers to a heterocyclic nitrogenous base in N-glycosidic linkage with a phosphorylated sugar. Nucleotides are recognized in the art to include natural bases (standard), and modified bases well known in the art. Such bases are generally located at the 1' position of a nucleotide sugar moiety. Nucleotides generally comprise a base, sugar and a phosphate group. The nucleotides can be unmodified or modified at the sugar, phosphate and/or base moiety, (also referred to interchangeably as nucleotide analogs, modified nucleotides, non-natural nucleotides, non-standard nucleotides and other; see for example, Usman and McSwiggen, *supra*; Eckstein *et al.*, International PCT Publication No. WO 92/07065; Usman *et al.*, International PCT Publication No. WO 93/15187; Uhlman & Peyman, supra all are hereby incorporated by reference herein. There are several examples of modified nucleic acid bases known in the art as summarized by Limbach *et al.*, 1994, Nucleic Acids Res. 22, 2183. Some of the non-limiting examples of chemically modified and other natural nucleic acid bases that can be introduced into nucleic acids include, for example, inosine, purine, pyridin-4-one, pyridin-2-one, phenyl, pseudouracil, 2, 4, 6-trimethoxy benzene, 3-methyl uracil, dihydrouridine, naphthyl, aminophenyl, 5-alkylcytidines (*e.g.*, 5-methylcytidine), 5-alkyluridines (*e.g.*, ribothymidine), 5-halouridine (*e.g.*, 5-bromouridine) or 6-azapyrimidines or 6-alkylpyrimidines (*e.g.* 6-methyluridine), propyne, quesosine, 2-thiouridine, 4-thiouridine, wybutosine, wybutoxosine, 4-acetylcytidine, 5-(carboxyhydroxymethyl)uridine, 5'-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluridine, beta-D-galactosylqueosine, 1-methyladenosine, 1-methylinosine, 2,2-dimethylguanosine, 3-methylcytidine, 2-methyladenosine, 2-methylguanosine, N6-methyladenosine, 7-methylguanosine, 5-methoxyaminomethyl-2-thiouridine, 5-methylaminomethyluridine, 5-methylcarbonylmethyluridine, 5-methyloxyuridine, 5-methyl-2-thiouridine, 2-methylthio-N6-isopentenyladenosine, beta-D-mannosylqueosine, uridine-5-oxyacetic acid, 2-thiocytidine, threonine derivatives and others (Burgin *et al.*, 1996, Biochemistry, 35, 14090; Uhlman & Peyman, *supra*). By "modified bases" in this aspect is meant nucleotide bases other than adenine, guanine, cytosine and uracil at 1' position or their equivalents; such bases can be used at any position, for example, within the catalytic core of an enzymatic nucleic acid molecule and/or in the substrate-binding regions of the nucleic acid molecule.

The term "nucleoside" as used herein refers to a heterocyclic nitrogenous base in N-glycosidic linkage with a sugar. Nucleosides are recognized in the art to include natural bases (standard), and modified bases well known in the art. Such bases are generally located at the 1' position of a nucleoside sugar moiety. Nucleosides generally comprise a base and sugar group. The nucleosides can be unmodified or modified at the sugar, and/or base moiety (also referred to interchangeably as nucleoside analogs, modified nucleosides, non-natural nucleosides, non-standard nucleosides and other; see for example, Usman and McSwiggen, *supra*; Eckstein *et al.,* International PCT Publication No. WO 92/07065; Usman *et al.,* International PCT Publication No. WO 93/15187; Uhlman & Peyman, supra all are hereby incorporated by reference herein). There are several examples of modified nucleic acid bases known in the art as summarized by Limbach *et al.,* 1994, Nucleic Acids Res. 22, 2183. Some of the non-limiting examples of chemically modified and other natural nucleic acid bases that can be introduced into nucleic acids include, inosine, purine, pyridin-4-one, pyridin-2-one, phenyl, pseudouracil, 2, 4, 6-trimethoxy benzene, 3-methyl uracil, dihydrouridine, naphthyl, aminophenyl, 5-alkylcytidines (*e.g.*, 5-methylcytidine), 5-alkyluridines (*e.g.*, ribothymidine), 5-halouridine (*e.g.*, 5-bromouridine) or 6-azapyrimidines or 6-alkylpyrimidines (*e.g.* 6-methyluridine), propyne, quesosine, 2-thiouridine, 4-thiouridine, wybutosine, wybutoxosine, 4-acetylcytidine, 5-(carboxyhydroxymethyl)uridine, 5'-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluridine, beta-D-galactosylqueosine, 1-methyladenosine, 1-methylinosine, 2,2-dimethylguanosine, 3-methylcytidine, 2-methyladenosine, 2-methylguanosine, N6-methyladenosine, 7-methylguanosine, 5-methoxyaminomethyl-2-thiouridine, 5-methylaminomethyluridine, 5-methylcarbonylmethyluridine, 5-methyloxyuridine, 5-methyl-2-thiouridine, 2-methylthio-N6-isopentenyladenosine, beta-D-mannosylqueosine, uridine-5-oxyacetic acid, 2-thiocytidine, threonine derivatives and others (Burgin *et al.,* 1996, Biochemistry, 35, 14090; Uhlman & Peyman, *supra*). By "modified bases" in this aspect is meant nucleoside bases other than adenine, guanine, cytosine and uracil at 1' position or their equivalents; such bases can be used at any position, for example, within the catalytic core of an enzymatic nucleic acid molecule and/or in the substrate-binding regions of the nucleic acid molecule.

In one embodiment, the invention features modified nucleic acid molecules with phosphate backbone modifications comprising one or more phosphorothioate, phosphorodithioate, methylphosphonate, morpholino, amidate carbamate, carboxymethyl, acetamidate, polyamide, sulfonate, sulfonamide, sulfamate, formacetal, thioformacetal, and/or alkylsilyl, substitutions. For a review of oligonucleotide backbone modifications see Hunziker and Leumann, 1995, *Nucleic Acid Analogues: Synthesis and Properties,* in *Modern Synthetic Methods,* VCH, 331-417, and Mesmaeker *et al.,* 1994, *Novel Backbone Replacements for Oligonucleotides,* in *Carbohydrate Modifications in Antisense Research,* ACS, 24-39. These references are hereby incorporated by reference herein.

The term "abasic" as used herein refers to sugar moieties lacking a base or having other chemical groups in place of a base at the 1' position, for example a 3',3'-linked or 5',5'-linked deoxyabasic ribose derivative (for more details see Wincott *et al.*, International PCT publication No. WO 97/26270).

The term "unmodified nucleoside" as used herein refers to one of the bases adenine, cytosine, guanine, thymine, uracil joined to the 1' carbon of β-D-ribo-furanose.

The term "modified nucleoside" as used herein refers to any nucleotide base which contains a modification in the chemical structure of an unmodified nucleotide base, sugar and/or phosphate.

In connection with 2'-modified nucleotides as described for the present invention, by "amino" is meant 2'-NH_2 or 2'-*O*- NH_2, which can be modified or unmodified. Such modified groups are described, for example, in Eckstein *et al.*, U.S. Patent 5,672,695 and Matulic-Adamic *et al.*, WO 98/28317, respectively, which are both incorporated by reference in their entireties.

Various modifications to nucleic acid (*e.g.*, enzymatic nucleic acid, antisense, decoy, aptamer, siRNA, triplex oligonucleotides, 2,5-A oligonucleotides and other nucleic acid molecules) structure can be made to enhance the utility of these molecules. For example, such modifications can enhance shelf life, half-life *in vitro*, stability, and ease of introduction of such oligonucleotides to the target site, including *e.g.*, enhancing penetration of cellular membranes and conferring the ability to recognize and bind to targeted cells.

Use of these molecules can lead to better treatment of the disease progression by affording the possibility of combination therapies (*e.g.*, multiple nucleic acid molecules targeted to different genes, nucleic acid molecules coupled with known small molecule inhibitors, or intermittent treatment with combinations of nucleic acid molecules (including different nucleic acid molecule motifs) and/or other chemical or biological molecules). The treatment of patients with nucleic acid molecules can also include combinations of different types of nucleic acid molecules. Therapies can be devised which include a mixture of enzymatic nucleic acid molecules (including different enzymatic nucleic acid molecule motifs), antisense, decoy, aptamer and/or 2-5A chimera molecules to one or more targets to alleviate symptoms of a disease.

Administration of Nucleic Acid Molecules

Methods for the delivery of nucleic acid molecules are described in Akhtar *et al.*, 1992, *Trends Cell Bio.*, 2, 139; *Delivery Strategies for Antisense Oligonucleotide Therapeutics*, ed. Akhtar, 1995, Maurer *et al.*, 1999, *Mol. Membr. Biol.*, 16, 129-140; Hofland and Huang,

1999, *Handb. Exp. Pharmacol.*, 137, 165-192; and Lee *et al.*, 2000, *ACS Symp. Ser.*, 752, 184-192, Sullivan *et al.*, PCT WO 94/02595, further describes the general methods for delivery of enzymatic nucleic acid molecules. These protocols can be utilized for the delivery of virtually any nucleic acid molecule. Nucleic acid molecules can be administered to cells by a variety of methods known to those of skill in the art, including, but not restricted to, encapsulation in liposomes, by iontophoresis, or by incorporation into other vehicles, such as hydrogels, cyclodextrins, biodegradable nanocapsules, and bioadhesive microspheres, or by proteinaceous vectors (O'Hare and Normand, International PCT Publication No. WO 00/53722). Alternatively, the nucleic acid/vehicle combination is locally delivered by direct injection or by use of an infusion pump. Direct injection of the nucleic acid molecules of the invention, whether subcutaneous, intramuscular, or intradermal, can take place using standard needle and syringe methodologies, or by needle-free technologies such as those described in Conry *et al.*, 1999, *Clin. Cancer Res.*, 5, 2330-2337 and Barry *et al.*, International PCT Publication No. WO 99/31262. The molecules of the instant invention can be used as pharmaceutical agents. Pharmaceutical agents prevent, modulate the occurrence, or treat (alleviate a symptom to some extent, preferably all of the symptoms) of a disease state in a patient.

Thus, the invention features a pharmaceutical composition comprising one or more nucleic acid(s) of the invention in an acceptable carrier, such as a stabilizer, buffer, and the like. The negatively charged polynucleotides of the invention can be administered (*e.g.*, RNA, DNA or protein) and introduced into a patient by any standard means, with or without stabilizers, buffers, and the like, to form a pharmaceutical composition. When it is desired to use a liposome delivery mechanism, standard protocols for formation of liposomes can be followed. The compositions of the present invention may also be formulated and used as tablets, capsules or elixirs for oral administration, suppositories for rectal administration, sterile solutions, suspensions for injectable administration, and the other compositions known in the art.

The present invention also includes pharmaceutically acceptable formulations of the compounds described. These formulations include salts of the above compounds, *e.g.*, acid addition salts, for example, salts of hydrochloric, hydrobromic, acetic acid, and benzene sulfonic acid.

A pharmacological composition or formulation refers to a composition or formulation in a form suitable for administration, *e.g.*, systemic administration, into a cell or patient, including for example a human. Suitable forms, in part, depend upon the use or the route of entry, for example oral, transdermal, or by injection. Such forms should not prevent the composition or formulation from reaching a target cell (*i.e.*, a cell to which the negatively

charged nucleic acid is desirable for delivery). For example, pharmacological compositions injected into the blood stream should be soluble. Other factors are known in the art, and include considerations such as toxicity and forms that prevent the composition or formulation from exerting its effect.

By "systemic administration" is meant *in vivo* systemic absorption or accumulation of drugs in the blood stream followed by distribution throughout the entire body. Administration routes which lead to systemic absorption include, without limitation: intravenous, subcutaneous, intraperitoneal, inhalation, oral, intrapulmonary and intramuscular. Each of these administration routes expose the desired negatively charged polymers, *e.g.*, nucleic acids, to an accessible diseased tissue. The rate of entry of a drug into the circulation has been shown to be a function of molecular weight or size. The use of a liposome or other drug carrier comprising the compounds of the instant invention can potentially localize the drug, for example, in certain tissue types, such as the tissues of the reticular endothelial system (RES). A liposome formulation that can facilitate the association of drug with the surface of cells, such as, lymphocytes and macrophages is also useful. This approach may provide enhanced delivery of the drug to target cells by taking advantage of the specificity of macrophage and lymphocyte immune recognition of abnormal cells, such as cancer cells.

By "pharmaceutically acceptable formulation" is meant, a composition or formulation that allows for the effective distribution of the nucleic acid molecules of the instant invention in the physical location most suitable for their desired activity. Nonlimiting examples of agents suitable for formulation with the nucleic acid molecules of the instant invention include: P-glycoprotein inhibitors (such as Pluronic P85), which can enhance entry of drugs into the CNS (Jolliet-Riant and Tillement, 1999, *Fundam. Clin. Pharmacol.*, 13, 16-26); biodegradable polymers, such as poly (DL-lactide-coglycolide) microspheres for sustained release delivery after intracerebral implantation (Emerich, DF *et al*, 1999, *Cell Transplant*, 8, 47-58) (Alkermes, Inc. Cambridge, MA); and loaded nanoparticles, such as those made of polybutylcyanoacrylate, which can deliver drugs across the blood brain barrier and can alter neuronal uptake mechanisms (*Prog Neuropsychopharmacol Biol Psychiatry*, 23, 941-949, 1999). Other non-limiting examples of delivery strategies for the nucleic acid molecules of the instant invention include material described in Boado *et al.*, 1998, *J. Pharm. Sci.*, 87, 1308-1315; Tyler *et al.*, 1999, *FEBS Lett.*, 421, 280-284; Pardridge *et al.*, 1995, *PNAS USA.*, 92, 5592-5596; Boado, 1995, *Adv. Drug Delivery Rev.*, 15, 73-107; Aldrian-Herrada *et al.*, 1998, *Nucleic Acids Res.*, 26, 4910-4916; and Tyler *et al.*, 1999, *PNAS USA.*, 96, 7053-7058.

The invention also features the use of the composition comprising surface-modified liposomes containing poly (ethylene glycol) lipids (PEG-modified, or long-circulating

liposomes or stealth liposomes). These formulations offer a method for increasing the accumulation of drugs in target tissues. This class of drug carriers resists opsonization and elimination by the mononuclear phagocytic system (MPS or RES), thereby enabling longer blood circulation times and enhanced tissue exposure for the encapsulated drug (Lasic *et al. Chem. Rev.* 1995, 95, 2601-2627; Ishiwata *et al., Chem. Pharm. Bull.* 1995, 43, 1005-1011). Such liposomes have been shown to accumulate selectively in tumors, presumably by extravasation and capture in the neovascularized target tissues (Lasic *et al., Science* 1995, 267, 1275-1276; Oku *et al.,*1995, *Biochim. Biophys. Acta*, 1238, 86-90). The long-circulating liposomes enhance the pharmacokinetics and pharmacodynamics of DNA and RNA, particularly compared to conventional cationic liposomes which are known to accumulate in tissues of the MPS (Liu *et al., J. Biol. Chem.* 1995, 42, 24864-24870; Choi *et al.,* International PCT Publication No. WO 96/10391; Ansell *et al.,* International PCT Publication No. WO 96/10390; Holland *et al.,* International PCT Publication No. WO 96/10392). Long-circulating liposomes are also likely to protect drugs from nuclease degradation to a greater extent compared to cationic liposomes, based on their ability to avoid accumulation in metabolically aggressive MPS tissues such as the liver and spleen.

The present invention also includes compositions prepared for storage or administration, which include a pharmaceutically effective amount of the desired compounds in a pharmaceutically acceptable carrier or diluent. Acceptable carriers or diluents for therapeutic use are well known in the pharmaceutical art, and are described, for example, in *Remington's Pharmaceutical Sciences*, Mack Publishing Co. (A.R. Gennaro edit. 1985) hereby incorporated by reference herein. For example, preservatives, stabilizers, dyes and flavoring agents may be provided. These include sodium benzoate, sorbic acid and esters of *p*-hydroxybenzoic acid. In addition, antioxidants and suspending agents may be used.

A pharmaceutically effective dose is that dose required to prevent, inhibit the occurrence of, or treat (alleviate a symptom to some extent, preferably all of the symptoms) a disease state. The pharmaceutically effective dose depends on the type of disease, the composition used, the route of administration, the type of mammal being treated, the physical characteristics of the specific mammal under consideration, concurrent medication, and other factors that those skilled in the medical arts will recognize. Generally, an amount between 0.1 mg/kg and 100 mg/kg body weight/day of active ingredients is administered dependent upon potency of the negatively charged polymer.

The present invention also includes compositions prepared for storage or administration that include a pharmaceutically effective amount of the desired compounds in a pharmaceutically acceptable carrier or diluent. Acceptable carriers or diluents for therapeutic use are well known in the pharmaceutical art, and are described, for example, in *Remington's*

Pharmaceutical Sciences, Mack Publishing Co. (A.R. Gennaro edit. 1985), hereby incorporated by reference herein. For example, preservatives, stabilizers, dyes and flavoring agents can be provided. These include sodium benzoate, sorbic acid and esters of *p*-hydroxybenzoic acid. In addition, antioxidants and suspending agents can be used.

A pharmaceutically effective dose is that dose required to prevent, inhibit the occurrence, or treat (alleviate a symptom to some extent, preferably all of the symptoms) of a disease state. The pharmaceutically effective dose depends on the type of disease, the composition used, the route of administration, the type of mammal being treated, the physical characteristics of the specific mammal under consideration, concurrent medication, and other factors that those skilled in the medical arts will recognize. Generally, an amount between 0.1 mg/kg and 100 mg/kg body weight/day of active ingredients is administered dependent upon potency of the negatively charged polymer.

The nucleic acid molecules of the invention and formulations thereof can be administered orally, topically, parenterally, by inhalation or spray, or rectally in dosage unit formulations containing conventional non-toxic pharmaceutically acceptable carriers, adjuvants and/or vehicles. The term parenteral as used herein includes percutaneous, subcutaneous, intravascular (*e.g.*, intravenous), intramuscular, or intrathecal injection or infusion techniques and the like. In addition, there is provided a pharmaceutical formulation comprising a nucleic acid molecule of the invention and a pharmaceutically acceptable carrier. One or more nucleic acid molecules of the invention can be present in association with one or more non-toxic pharmaceutically acceptable carriers and/or diluents and/or adjuvants, and if desired other active ingredients. The pharmaceutical compositions containing nucleic acid molecules of the invention can be in a form suitable for oral use, for example, as tablets, troches, lozenges, aqueous or oily suspensions, dispersible powders or granules, emulsion, hard or soft capsules, or syrups or elixirs.

Compositions intended for oral use can be prepared according to any method known to the art for the manufacture of pharmaceutical compositions and such compositions can contain one or more such sweetening agents, flavoring agents, coloring agents or preservative agents in order to provide pharmaceutically elegant and palatable preparations. Tablets contain the active ingredient in admixture with non-toxic pharmaceutically acceptable excipients that are suitable for the manufacture of tablets. These excipients can be, for example, inert diluents; such as calcium carbonate, sodium carbonate, lactose, calcium phosphate or sodium phosphate; granulating and disintegrating agents, for example, corn starch, or alginic acid; binding agents, for example starch, gelatin or acacia; and lubricating agents, for example magnesium stearate, stearic acid or talc. The tablets can be uncoated or they can be coated by known techniques. In some cases such coatings can be prepared by

known techniques to delay disintegration and absorption in the gastrointestinal tract and thereby provide a sustained action over a longer period. For example, a time delay material such as glyceryl monosterate or glyceryl distearate can be employed.

Formulations for oral use can also be presented as hard gelatin capsules wherein the active ingredient is mixed with an inert solid diluent, for example, calcium carbonate, calcium phosphate or kaolin, or as soft gelatin capsules wherein the active ingredient is mixed with water or an oil medium, for example peanut oil, liquid paraffin or olive oil.

Aqueous suspensions contain the active materials in admixture with excipients suitable for the manufacture of aqueous suspensions. Such excipients are suspending agents, for example sodium carboxymethylcellulose, methylcellulose, hydropropyl-methylcellulose, sodium alginate, polyvinylpyrrolidone, gum tragacanth and gum acacia; dispersing or wetting agents can be a naturally-occurring phosphatide, for example, lecithin, or condensation products of an alkylene oxide with fatty acids, for example polyoxyethylene stearate, or condensation products of ethylene oxide with long chain aliphatic alcohols, for example heptadecaethyleneoxycetanol, or condensation products of ethylene oxide with partial esters derived from fatty acids and a hexitol such as polyoxyethylene sorbitol monooleate, or condensation products of ethylene oxide with partial esters derived from fatty acids and hexitol anhydrides, for example polyethylene sorbitan monooleate. The aqueous suspensions can also contain one or more preservatives, for example ethyl, or n-propyl p-hydroxybenzoate, one or more coloring agents, one or more flavoring agents, and one or more sweetening agents, such as sucrose or saccharin.

Oily suspensions can be formulated by suspending the active ingredients in a vegetable oil, for example arachis oil, olive oil, sesame oil or coconut oil, or in a mineral oil such as liquid paraffin. The oily suspensions can contain a thickening agent, for example beeswax, hard paraffin or cetyl alcohol. Sweetening agents and flavoring agents can be added to provide palatable oral preparations. These compositions can be preserved by the addition of an anti-oxidant such as ascorbic acid.

Dispersible powders and granules suitable for preparation of an aqueous suspension by the addition of water provide the active ingredient in admixture with a dispersing or wetting agent, suspending agent and one or more preservatives. Suitable dispersing or wetting agents or suspending agents are exemplified by those already mentioned above. Additional excipients, for example sweetening, flavoring and coloring agents, can also be present.

Pharmaceutical compositions of the invention can also be in the form of oil-in-water emulsions. The oily phase can be a vegetable oil or a mineral oil or mixtures of these. Suitable emulsifying agents can be naturally-occurring gums, for example gum acacia or gum

tragacanth, naturally-occurring phosphatides, for example soy bean, lecithin, and esters or partial esters derived from fatty acids and hexitol, anhydrides, for example sorbitan monooleate, and condensation products of the said partial esters with ethylene oxide, for example polyoxyethylene sorbitan monooleate. The emulsions can also contain sweetening and flavoring agents.

Syrups and elixirs can be formulated with sweetening agents, for example glycerol, propylene glycol, sorbitol, glucose or sucrose. Such formulations can also contain a demulcent, a preservative and flavoring and coloring agents. The pharmaceutical compositions can be in the form of a sterile injectable aqueous or oleaginous suspension. This suspension can be formulated according to the known art using those suitable dispersing or wetting agents and suspending agents that have been mentioned above. The sterile injectable preparation can also be a sterile injectable solution or suspension in a non-toxic parentally acceptable diluent or solvent, for example as a solution in 1,3-butanediol. Among the acceptable vehicles and solvents that can be employed are water, Ringer's solution and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose, any bland fixed oil can be employed including synthetic mono-or diglycerides. In addition, fatty acids such as oleic acid find use in the preparation of injectables.

The nucleic acid molecules of the invention can also be administered in the form of suppositories, *e.g.*, for rectal administration of the drug. These compositions can be prepared by mixing the drug with a suitable non-irritating excipient that is solid at ordinary temperatures but liquid at the rectal temperature and will therefore melt in the rectum to release the drug. Such materials include cocoa butter and polyethylene glycols.

Nucleic acid molecules of the invention can be administered parenterally in a sterile medium. The drug, depending on the vehicle and concentration used, can either be suspended or dissolved in the vehicle. Advantageously, adjuvants such as local anesthetics, preservatives and buffering agents can be dissolved in the vehicle.

Dosage levels of the order of from about 0.1 mg to about 140 mg per kilogram of body weight per day are useful in the treatment of the above-indicated conditions (about 0.5 mg to about 7 g per patient per day). The amount of active ingredient that can be combined with the carrier materials to produce a single dosage form varies depending upon the host treated and the particular mode of administration. Dosage unit forms generally contain between from about 1 mg to about 500 mg of an active ingredient.

It is understood that the specific dose level for any particular patient depends upon a variety of factors including the activity of the specific compound employed, the age, body

weight, general health, sex, diet, time of administration, route of administration, and rate of excretion, drug combination and the severity of the particular disease undergoing therapy.

For administration to non-human animals, the composition can also be added to the animal feed or drinking water. It can be convenient to formulate the animal feed and drinking water compositions so that the animal takes in a therapeutically appropriate quantity of the composition along with its diet. It can also be convenient to present the composition as a premix for addition to the feed or drinking water.

The nucleic acid molecules of the present invention may also be administered to a patient in combination with other therapeutic compounds to increase the overall therapeutic effect. The use of multiple compounds to treat an indication may increase the beneficial effects while reducing the presence of side effects.

In one embodiment, the invention compositions suitable for administering nucleic acid molecules of the invention to specific cell types, such as hepatocytes. For example, the asialoglycoprotein receptor (ASGPr) (Wu and Wu, 1987, *J. Biol. Chem.* 262, 4429-4432) is unique to hepatocytes and binds branched galactose-terminal glycoproteins, such as asialoorosomucoid (ASOR). Binding of such glycoproteins or synthetic glycoconjugates to the receptor takes place with an affinity that strongly depends on the degree of branching of the oligosaccharide chain, for example, triatennary structures are bound with greater affinity than biatenarry or monoatennary chains (Baenziger and Fiete, 1980, *Cell,* 22, 611-620; Connolly *et al.,* 1982, *J. Biol. Chem.,* 257, 939-945). Lee and Lee, 1987, *Glycoconjugate J.,* 4, 317-328, obtained this high specificity through the use of N-acetyl-D-galactosamine as the carbohydrate moiety, which has higher affinity for the receptor, compared to galactose. This "clustering effect" has also been described for the binding and uptake of mannosyl-terminating glycoproteins or glycoconjugates (Ponpipom *et al.,* 1981, *J. Med. Chem.,* 24, 1388-1395). The use of galactose and galactosamine based conjugates to transport exogenous compounds across cell membranes can provide a targeted delivery approach to the treatment of liver disease such as HBV infection or hepatocellular carcinoma. The use of bioconjugates can also provide a reduction in the required dose of therapeutic compounds required for treatment. Furthermore, therapeutic bioavialability, pharmacodynamics, and pharmacokinetic parameters can be modulated through the use of nucleic acid bioconjugates of the invention.

Alternatively, certain of the nucleic acid molecules of the instant invention can be expressed within cells from eukaryotic promoters (*e.g.,* Izant and Weintraub, 1985, *Science,* 229, 345; McGarry and Lindquist, 1986, *Proc. Natl. Acad. Sci.,* USA 83, 399; Scanlon *et al.,* 1991, *Proc. Natl. Acad. Sci. USA,* 88, 10591-5; Kashani-Sabet *et al.,* 1992, *Antisense Res. Dev.,* 2, 3-15; Dropulic *et al.,* 1992, *J. Virol.,* 66, 1432-41; Weerasinghe *et al.,* 1991, *J. Virol.,* 65, 5531-4; Ojwang *et al.,* 1992, *Proc. Natl. Acad. Sci. USA,* 89, 10802-6; Chen *et*

al., 1992, *Nucleic Acids Res.*, 20, 4581-9; Sarver *et al.*, 1990 *Science*, 247, 1222-1225; Thompson *et al.*, 1995, *Nucleic Acids Res.*, 23, 2259; Good *et al.*, 1997, *Gene* Therapy, 4, 45; all of these references are hereby incorporated in their totalities by reference herein). Those skilled in the art realize that any nucleic acid can be expressed in eukaryotic cells from the appropriate DNA/RNA vector. The activity of such nucleic acids can be augmented by their release from the primary transcript by a ribozyme (Draper *et al.*, PCT WO 93/23569, and Sullivan *et al.*, PCT WO 94/02595; Ohkawa *et al.*, 1992, *Nucleic Acids Symp. Ser.*, 27, 15-6; Taira *et al.*, 1991, *Nucleic Acids Res.*, 19, 5125-30; Ventura *et al.*, 1993, *Nucleic Acids Res.*, 21, 3249-55; Chowrira *et al.*, 1994, *J. Biol. Chem.*, 269, 25856; all of these references are hereby incorporated in their totality by reference herein).

In another aspect of the invention, RNA molecules of the present invention are preferably expressed from transcription units (see, for example, Couture *et al.*, 1996, *TIG.*, 12, 510) inserted into DNA or RNA vectors. The recombinant vectors are preferably DNA plasmids or viral vectors. Ribozyme expressing viral vectors could be constructed based on, but not limited to, adeno-associated virus, retrovirus, adenovirus, or alphavirus. Preferably, the recombinant vectors capable of expressing the nucleic acid molecules are delivered as described above, and persist in target cells. Alternatively, viral vectors may be used that provide for transient expression of nucleic acid molecules. Such vectors might be repeatedly administered as necessary. Once expressed, the nucleic acid molecule binds to the target mRNA. Delivery of nucleic acid molecule expressing vectors could be systemic, such as by intravenous or intra-muscular administration, by administration to target cells ex-planted from the patient followed by reintroduction into the patient, or by any other means that would allow for introduction into the desired target cell (for a review see Couture *et al.*, 1996, *TIG.*, 12, 510).

In one aspect, the invention features an expression vector comprising a nucleic acid sequence encoding at least one of the nucleic acid molecules of the instant invention is disclosed. The nucleic acid sequence encoding the nucleic acid molecule of the instant invention is operable linked in a manner which allows expression of that nucleic acid molecule.

In another aspect the invention features an expression vector comprising: a) a transcription initiation region (*e.g.*, eukaryotic pol I, II or III initiation region); b) a transcription termination region (*e.g.*, eukaryotic pol I, II or III termination region); c) a nucleic acid sequence encoding at least one of the nucleic acid catalyst of the instant invention; and wherein said sequence is operably linked to said initiation region and said termination region, in a manner which allows expression and/or delivery of said nucleic acid molecule. The vector may optionally include an open reading frame (ORF) for a protein

operably linked on the 5' side or the 3'-side of the sequence encoding the nucleic acid catalyst of the invention; and/or an intron (intervening sequences).

Transcription of the nucleic acid molecule sequences are driven from a promoter for eukaryotic RNA polymerase I (pol I), RNA polymerase II (pol II), or RNA polymerase III (pol III). Transcripts from pol II or pol III promoters will be expressed at high levels in all cells; the levels of a given pol II promoter in a given cell type will depend on the nature of the gene regulatory sequences (enhancers, silencers, etc.) present nearby. Prokaryotic RNA polymerase promoters are also used, providing that the prokaryotic RNA polymerase enzyme is expressed in the appropriate cells (Elroy-Stein and Moss, 1990, *Proc. Natl. Acad. Sci. U S A*, 87, 6743-7; Gao and Huang 1993, *Nucleic Acids Res.*, 21, 2867-72; Lieber et al., 1993, *Methods Enzymol.*, 217, 47-66; Zhou et al., 1990, *Mol. Cell. Biol.*, 10, 4529-37). All of these references are incorporated by reference herein. Several investigators have demonstrated that nucleic acid molecules, such as ribozymes expressed from such promoters can function in mammalian cells (e.g. Kashani-Sabet et al., 1992, *Antisense Res. Dev.*, 2, 3-15; Ojwang et al., 1992, *Proc. Natl. Acad. Sci. U S A*, 89, 10802-6; Chen *et al.*, 1992, *Nucleic Acids Res.*, 20, 4581-9; Yu et al., 1993, *Proc. Natl. Acad. Sci. U S A*, 90, 6340-4; L'Huillier *et al.*, 1992, *EMBO J.*, 11, 4411-8; Lisziewicz *et al.*, 1993, *Proc. Natl. Acad. Sci. U. S. A*, 90, 8000-4; Thompson *et al.*, 1995, *Nucleic Acids Res.*, 23, 2259; Sullenger & Cech, 1993, *Science*, 262, 1566). More specifically, transcription units such as the ones derived from genes encoding U6 small nuclear (snRNA), transfer RNA (tRNA) and adenovirus VA RNA are useful in generating high concentrations of desired RNA molecules such as ribozymes in cells (Thompson *et al., supra*; Couture and Stinchcomb, *1996, supra*; Noonberg *et al.*, 1994, *Nucleic Acid Res.*, 22, 2830; Noonberg *et al.*, US Patent No. 5,624,803; Good *et al.*, 1997, *Gene Ther.*, 4, 45; Beigelman *et al.*, International PCT Publication No. *WO 96/18736*; all of these publications are incorporated by reference herein). The above ribozyme transcription units can be incorporated into a variety of vectors for introduction into mammalian cells, including but not restricted to, plasmid DNA vectors, viral DNA vectors (such as adenovirus or adeno-associated virus vectors), or viral RNA vectors (such as retroviral or alphavirus vectors) (for a review see Couture and Stinchcomb, *1996, supra).*

In yet another aspect, the invention features an expression vector comprising nucleic acid sequence encoding at least one of the nucleic acid molecules of the invention, in a manner that allows expression of that nucleic acid molecule. The expression vector comprises in one embodiment; a) a transcription initiation region; b) a transcription termination region; c) a nucleic acid sequence encoding at least one said nucleic acid molecule; and wherein said sequence is operably linked to said initiation region and said termination region, in a manner which allows expression and/or delivery of said nucleic acid molecule. In another embodiment, the expression vector comprises: a) a transcription initiation region; b) a